Sensors and Actuators B 235 (2016) 563–567

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Colorimetric detection of active botulinum neurotoxin using Cu^{2+} mediated gold nanoparticles agglomeration

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ARTICLE INFO

Article history:
Received 23 January 2016
Received in revised form 5 May 2016
Accepted 24 May 2016
Available online 24 May 2016

Keywords:
Colorimetric
Botulinum neurotoxin
Gold nanoparticles

ABSTRACT

We report a colorimetric detection of functional botulinum neurotoxin (BoNT) based on its proteolytic activity. Active BoNT can enzymatically cleave SNAptide, a short peptide with 15 amino acids. The cleaved peptides subsequently induced the agglomeration of gold nanoparticles, allowing a colorimetric signal that can be discerned by the naked eye at the low concentration of 1 ng/mL (6.67 pM), and achieve low limit of detection at 0.25 ng/mL (1.67 pM 3 σ/s) by spectral absorbance. This colorimetric detection will provide a simple and promising method for applications at low-resource sites.

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1. Introduction

Botulinum neurotoxin (BoNT) produced by *Clostridium Botulinum* is considered one of the world's most dangerous toxins [1]. Intoxication by BoNT may occur through the ingestion of contaminated food or from biological weapons in some undeveloped countries or military areas. Due to its toxicity, remarkable stability, and persistence in the body, a very low dose of BoNT can cause muscle paralysis or even death [2,3], in which the lethal dose for humans is estimated to be about 1–2 ng per kg of body weight [4]. Therefore an extremely sensitive method of detecting BoNT is necessary. Conventional methods for detecting BoNT have been reported, such as mouse bioassay [5], ELISA [6], surface plasma resonance (SPR) immunoassay [7], and electrochemical luminescence immunoassay [8]. However, observing symptoms in live animal after injecting BoNT is very time-consuming and may raise ethical concerns. Immunoassays can reach a low limit of detection (LOD), but require complicated operations and cumbersome instrumentation for signal readouts. More importantly, immunoassays may only reflect the total amount of both active and inactive toxin. False positive results may be given even if only inactive toxin is present, posing uncertainty of the assays.

The light chain of BoNT has a proteolytic function, which can degrade components of the soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) and subsequently cause muscle paralysis [9,10]. Colorimetric [11], fluorescence [12], and luminescence [13] assays were introduced to detect the BoNT light chain based on the function, in which SNAPtide, a short peptide, was designed to mimic the synaptosomal-associated protein 25 (SNAP-25, a component of the trans-SNARE complex) with a cleavable site [14,15]. However, the methods are constrained by either the LOD (0.1 nM with a colorimetric signal) or the requirement of specific optical detectors (fluorescence or luminescence), making them unsuitable for practical point-of-care applications. Based on the proteolytic function of BoNT, here we report a colorimetric detection of active BoNT using an agglomeration of gold nanoparticles (AuNPs). AuNPs have been widely used as color indicator to detect DNA [16–18], protein [19–22], organic molecules [23], and metal ions [24–26], due to their unique property of changing color from red to purple after agglomeration [27]. As shown in Fig. 1, active BoNT first cleaved SNAPtides at step 1, and then magnetic microparticles modified with streptavidin were introduced to remove the cleaved peptide fragments with biotin ends via magnetic separation at step 2. At step 3, AuNPs were added, and the remaining peptide fragments with cysteine ends were immobilized onto AuNPs via the thiol group. At step 4, amino acid units such as arginine and asparagine in the cleaved peptide fragments

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http://dx.doi.org/10.1016/j.snb.2016.05.118
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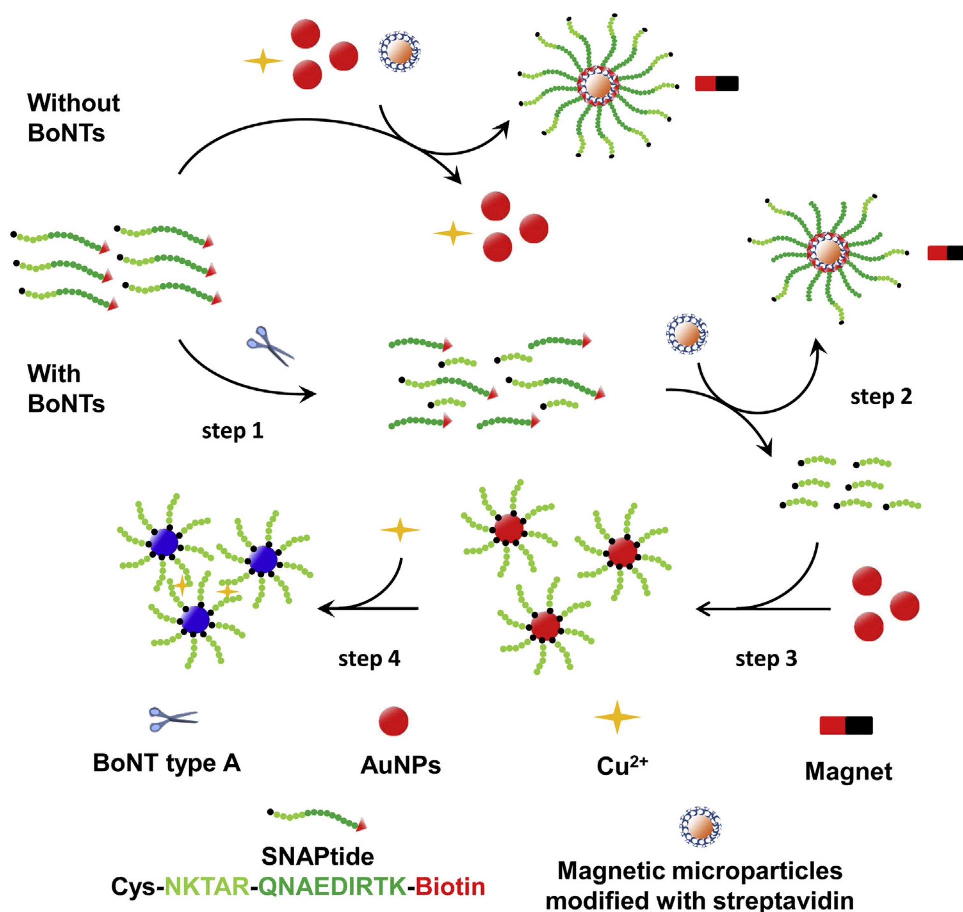

Fig. 1. Working principle of the colorimetric detection of active botulinum neurotoxin (BoNT) using gold nanoparticles (AuNPs). SNAPtide has biotin and cysteine on its two termini. When BoNT was absent (upper pathway), magnetic microparticles modified with streptavidin were used to remove all SNAPtide and the AuNPs remained mono-dispersed. When BoNT was present (middle to lower pathway), SNAPtide was enzymatically cleaved by BoNT at step 1. At step 2, the fragments with the biotin end were removed using magnetic microparticles. At step 3, AuNPs were added to bind with the cleaved peptide with a cysteine end. At step 4, Cu^{2+} was used to link the free amine groups on the peptide fragments, leading to the agglomeration of AuNPs.

contain free amine groups that easily chelate with Cu^{2+} and result in the agglomeration of AuNPs. We found that the LOD reached 0.25 ng/mL (1.67 pM, $3\sigma/s$), which is to our knowledge the most sensitive colorimetric detection based on BoNT activity. This greatly improved sensitivity of our approach has potential for the on-site detection of BoNT in resource-limited contexts.

2. Experimental

The experimental procedures are provided in the Supplementary material.

3. Results

We first tested the proteolytic activity of BoNT for cleaving the SNAPtide using matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry. The SNAPtide designed for the MALDI-TOF mass spectrometry test was biotinylated on its both ends (see S1.1 of the Supplementary material) to increase the molecular weight of the cleaved peptide to over 800 Da and minimize interference from the matrix ions. The SNAPtide can be seen from the peak of 2225.2 (Fig. 2(A)). After incubation with BoNT, two new peaks at 942.553 and 1300.63 (Fig. 2(B)) appeared, which correspond to the molecular weight of the two cleaved peptides (see S1.1 of the Supplementary material). Therefore, the result confirmed that the BoNT used in this assay did have the proteolytic function to cleave the SNAPtide.

We next tested the feasibility of the colorimetric assay (see S1.2 and S1.3 of the Supplementary material). In the absence of BoNT (Buffer + peptide, Fig. 3) at step 1, the resulting AuNPs solution remained red, indicating that the non-cleaved peptide was removed by the magnetic microparticles. More importantly, the addition of Cu^{2+} did not cause the non-specific agglomeration of the unmodified AuNPs, even though they can be unstable when the surrounding ionic strength is changed. This is due to the use of the HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), which has a moderate affinity to bind with Cu^{2+}. After mixing with the AuNPs, the concentration of HEPES became 8.3 mM (see S1.3 of the Supplementary material), which is much greater than the concentration of added Cu^{2+} (1.6 mM). Thus, the added Cu^{2+} at the final step was captured by HEPES and did not lead to the non-specific agglomeration of the AuNPs (Fig. S1). In contrast, when using 1 μg/mL BoNT at step 1 the color of the AuNPs solution changed from red to light blue with the addition of Cu^{2+} (Peptide + BoNT, Fig. 3), suggesting that the amine group on the peptide fragment competes with the hydroxyl group and the sulfonic group of HEPES, forming a chemical complex with Cu^{2+} due to its stronger affinity, eventually causing the agglomeration of the AuNPs (Fig. S1).

BoNT is also a protein containing many amine and hydroxyl groups, so we explored the possibility of non-specific agglomeration caused by BoNT. At step 1, 1 μg/mL BoNT was used without SNAPtide and the AuNP solution remained red after the addition of Cu^{2+} (Buffer + BoNT, Fig. 3), indicating that BoNT does not cause

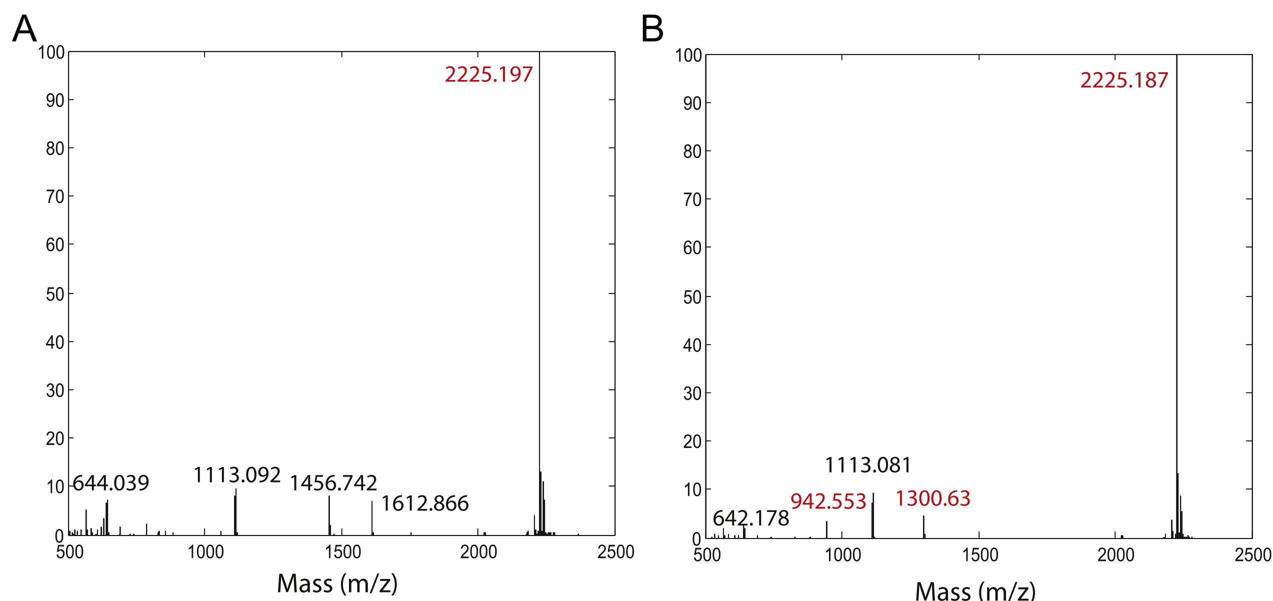

Fig. 2. The test of proteolytic activity of BoNT by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry. (A) 10 μM of SNAPtide. (B) 10 μM of SNAPtide solution after incubating with 5 μg/mL BoNT.

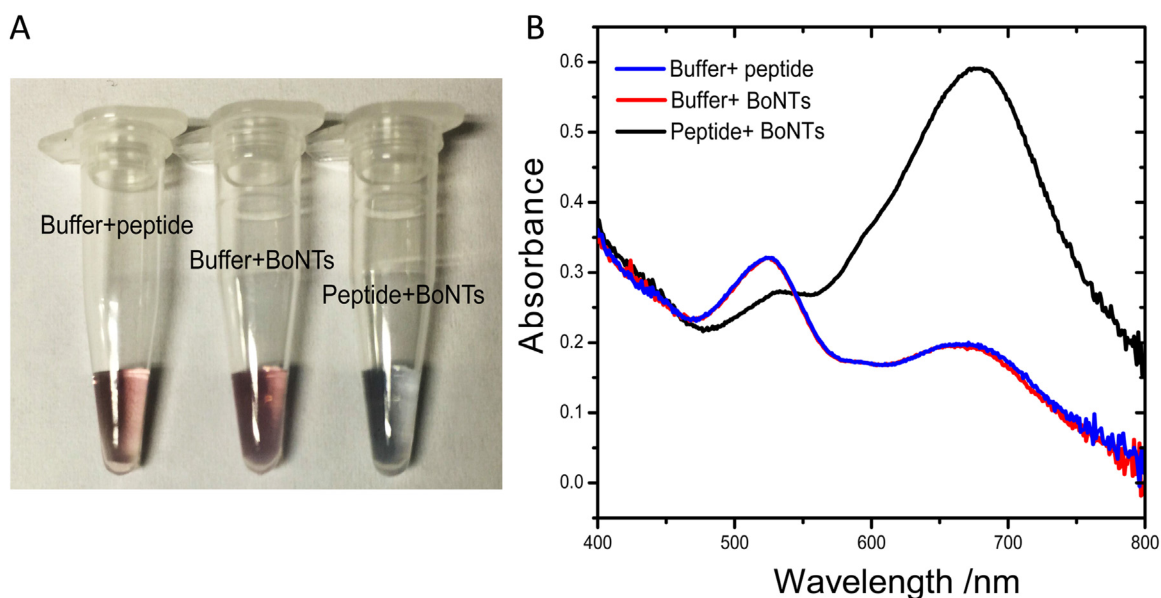

Fig. 3. The feasibility test of the colorimetric assay. (A) An optical image showing the color change of AuNP solution when buffer + SNAPtide, buffer + 1 μg/mL BoNT, or SNAPtide + 1 μg/mL BoNT was used in step 1. (B) The spectral absorbance of the solutions in (A).

non-specific agglomeration. We suspect that the BoNT structure is more complicated than the cleaved peptide, so the Cu^{2+} is more likely to embed in the folded structure, rather than forming a chemical complex connecting multiple BoNT.

To investigate selectivity, we used a 10% bovine serum, which contains enriched proteins and enzymes that may cleave the SNAPtide or introduce the non-specific agglomeration of AuNPs. While the AuNP solution changed from red to light blue due to the presence of 1 μg/mL BoNT, the 10% bovine serum only slightly changed the color of AuNP solution from red to pink (Fig. S2), suggesting that our assay is selective to BoNT and free from other non-specific interference.

We then explored the LOD of this method. With a varied concentration of BoNT, the color of the AuNP solution gradually changed from red to light blue (Fig. 4(A) and (B)). The color change can be discerned by the naked eye at the low concentration of 1 ng/mL (6.67 pM). With a linear range from 10 ng/mL to 1 μg/mL, the calibration equation was determined as $AR = -0.26198\,\lg(C) + 0.41971$, where AR is the absorbance ratio A520/A680, C is the concentration of BoNT, and the corresponding correlation coefficient (R^2) of the calibration curve is 0.985 (Fig. 4(C)). From the calibration graph, we found that the LOD reached 0.25 ng/mL (1.67 pM, calculated based on $3\sigma/s$, where σ is the standard deviation of lg(C) of control sample, and s is the absolute slope of the calibration equation), which is about 60 times lower than the LOD of other colorimetric detection based on BoNT activity (0.1 nM) [10], and comparable with the LOD of fluorescence assays (1 pM) [11]. The lethal range of BoNT to the human body is 1–2 ng/kg, which equals 60–120 ng for an adult (60 kg) and 25–50 ng for a child (25 kg). The LOD of our assay is

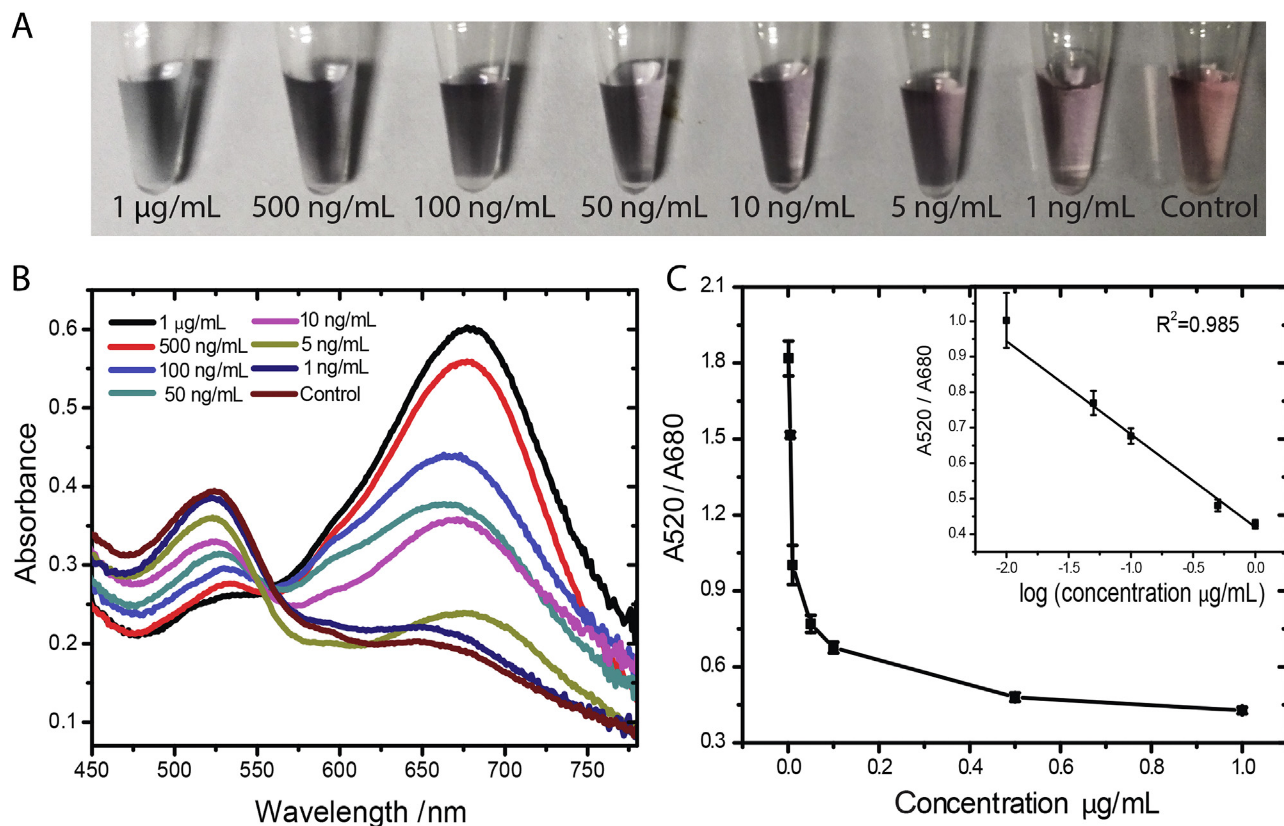

Fig. 4. Colorimetric detection of active BoNT with varying concentrations. (A) Optical images showing the change of solution color. Control, 0 ng/mL of BoNT. (B) Spectral absorbance of the AuNP solution. (C) Absorbance ratio (A520/A680) of the AuNP solution (mean ± max deviation, n = 3).

0.25 ng/mL, so it is able to determine 25 ng of BoNTs in a 100 mL sample, showing the potential for practical applications.

The improvement in sensitivity is owing to the use of Cu^{2+} and peptide fragments. In other colorimetric detection of BoNT activity [10], the AuNPs were linked using SNAPtide, and the linkage was broken due to the enzymatic cleavage of functional BoNT. This "turn-off" detection requires a large amount of BoNT to dissociate the AuNPs and to obtain the corresponding change in solution color, making it insensitive to low concentrations of BoNT. Compared to it, our "turn-on" detection of active BoNT relies on only few copies of peptide fragments when a low concentration of active BoNT was used. The Cu^{2+} has a strong affinity to form a chemical complex with the amine groups on the peptide fragments. Thus, rather than using the peptide as a bridge to connect multiple AuNPs and then break it, using Cu^{2+} and the peptide fragment is more efficient for inducing AuNP agglomeration in the presence of BoNT, enabling the colorimetric detection of active BoNT with a LOD of 1.67 pM.

4. Conclusion

We have developed a highly sensitive colorimetric method of detecting active BoNT based on the BoNT cleaved peptides, which results in the agglomeration of AuNPs and changes the solution color. Because each peptide fragment is fully utilized, this method allows for visual inspection at a low concentration of BoNT, 1 ng/mL (6.67 pM) and significantly improved LOD at 0.25 ng/mL (1.67 pM, $3\sigma/s$). Thus, the method has the advantages of simplicity and high sensitivity, providing broad and promising applications for the detection of BoNT at low-resource sites.

Acknowledgements

We are pleased to acknowledge the support of the National Natural Science Foundation of China (Grant No. 51305375), the Idea Incubator Scheme (Grant No. 6987032) from the City University of Hong Kong, the Early Career Scheme of the Hong Kong Research Grant Council (project no. 21214815), and the Science Technology and Innovation Committee of Shenzhen Municipality (Grant No. JCYJ20150601102053061).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2016.05.118.

References

[1] C. Montecucco, J. Molgo, Botulinal neurotoxins: revival of an old killer, Curr. Opin. Pharm. 5 (2005) 274–279.
[2] F.J. Erbguth, Historical notes on botulism, Clostridium batulinum, botulinum toxin, and the idea of the therapeutic use of the toxin, Mov. Disord. 19 (2004) S2–S6.
[3] C. Lamanna, The most poisonous poison, Science 130 (1959) 763–772.
[4] S.S. Arnon, R. Schechter, T.V. Inglesby, D.A. Henderson, J.G. Bartlett, M.S. Ascher, et al., Botulinum toxin as a biological weapon-medical and public health management, J. Am. Med. Assoc. 285 (2001) 1059–1070.
[5] S.K. Sharma, B.S. Eblen, R.L. Bull, D.H. Burr, R.C. Whiting, Evaluation of lateral-flow Clostridium botulinum neurotoxin detection kits for food analysis, Appl. Environ. Microb. 71 (2005) 3935–3941.
[6] M.A. Poli, V.R. Rivera, D. Neal, Development of sensitive colorimetric capture ELISAs for Clostridium botulinum neurotoxin serotypes E and F, Toxicon 40 (2002) 797–802.
[7] J. Ladd, A.D. Taylor, J. Homola, S.Y. Jiang, Detection of botulinum neurotoxins in buffer and honey using a surface plasmon resonance (SPR) sensor, Sens. Actuators B Chem. 130 (2008) 129–134.
[8] V.R. Rivera, F.J. Gamez, W.K. Keener, J.A. White, M.A. Poli, Rapid detection of Clostridium botulinum toxins A, B, E, and F in clinical samples, selected food

matrices, and buffer using paramagnetic bead-based electrochemiluminescence detection, Anal. Biochem. 353 (2006) 248–256.
[9] B.R. Singh, Botulinum neurotoxin structure engineering, and novel cellular trafficking and targeting, Neurotox. Res. 9 (2006) 73–92.
[10] P.G. Foran, N. Mohammed, G.O. Lisk, S. Nagwaney, G.W. Lawrence, E. Johnson, et al., Evaluation of the therapeutic usefulness of botulinum neurotoxin B, C1, E, and F compared with the long lasting type A. Basis for distinct durations of inhibition of exocytosis in central neurons, J. Biol. Chem. 278 (2003) 1363–1371.
[11] X.H. Liu, Y. Wang, P. Chen, Y.S. Wang, J.L. Mang, D. Aili, et al., Biofunctionalized gold nanoparticles for colorimetric sensing of botulinum neurotoxin: a light chain, Anal. Chem. 86 (2014) 2345–2352.
[12] Y. Wang, X.H. Liu, J.L. Zhang, D. Aili, B. Liedberg, Time-resolved botulinum neurotoxin: an activity monitored using peptide-functionalized Au nanoparticle energy transfer sensors, Chem. Sci. 5 (2014) 2651–2656.
[13] G.B. Stevens, D.A. Silver, A. Zgaga-Griesz, W.G. Bessler, S.K. Vashist, P. Patel, et al., Bioluminescence assay for the highly sensitive detection of botulinum neurotoxin A activity, Analyst 138 (2013) 6154–6162.
[14] J.J. Schmidt, K.A. Bostian, Endoproteinase activity of type A botulinum neurotoxin: substrate requirements and activation by serum albumin, J. Protein Chem. 16 (1997) 19–26.
[15] J.J. Schmidt, K.A. Bostian, Proteolysis of synthetic peptides by type A botulinum neurotoxin, J. Protein Chem. 14 (1995) 703–708.
[16] R. Elghanian, J.J. Storhoff, R.C. Mucic, R.L. Letsinger, C.A. Mirkin, Selective colorimetric detection of polynucleotides based on the distance-dependent optical properties of gold nanoparticles, Science 277 (1997) 1078–1081.
[17] Z.Q. Yuan, J. Cheng, X.D. Cheng, Y. He, E.S. Yeung, Highly sensitive DNA hybridization detection with single nanoparticle flash-lamp darkfield microscopy, Analyst 137 (2012) 2930–2932.
[18] R. Kanjanawarut, X.D. Su, Colorimetric detection of DNA using unmodified metallic nanoparticles and peptide nucleic acid probes, Anal. Chem. 81 (2009) 6122–6129.
[19] F. Wei, R. Lam, S. Cheng, S. Lu, D. Ho, N. Li, Rapid detection of melamine in whole milk mediated by unmodified gold nanoparticles, Appl. Phys. Lett. 96 (2010) 133702.
[20] F. Xia, X.L. Zuo, R.Q. Yang, Y. Xiao, D. Kang, A. Vallée-Bélisleb, et al., Colorimetric detection of DNA small molecules, proteins, and ions using unmodified gold nanoparticles and conjugated polyelectrolytes, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 10837–10841.
[21] Y. Wang, D. Zhang, W. Liu, X. Zhang, S. Yu, T. Liu, et al., Facile colorimetric method for simple and rapid detection of endotoxin based on counterion-mediated gold nanorods aggregation, Biosens. Bioelectron. 55 (2014) 242–248.
[22] F. Zhang, J. Zhu, J.J. Li, J.W. Zhao, A promising direct visualization of an Au@Ag nanorod-based colorimetric sensor for trace detection of alpha-fetoprotein, J. Mater. Chem. C 3 (2015) 6035–6045.
[23] J.M. Lin, Y.Q. Huang, Z.b. Liu, C.Q. Lin, X. Ma, J.M. Liu, Design of an ultra-sensitive gold nanorod colorimetric sensor and its application based on formaldehyde reducing Ag+, RSC Adv. 5 (2015) 99944–99950.
[24] S. Chen, Y.M. Fang, Q. Xiao, J. Li, S.B. Li, H.J. Chen, et al., Rapid visual detection of aluminium ion using citrate capped gold nanoparticles, Analyst 137 (2012) 2021–2023.
[25] Y.M. Fang, J. Song, J.S. Chen, S.B. Li, L. Zhang, G.N. Chen, et al., Gold nanoparticles for highly sensitive and selective copper ions sensing-old materials with new tricks, J. Mater. Chem. 21 (2011) 7898–7900.
[26] H.H. Cai, D. Lin, J. Wang, P.H. Yang, J. Cai, Controlled side-by-side assembly of gold nanorods: a strategy for lead detection, Sens. Actuators B Chem. 196 (2014) 252–259.
[27] U. Kreibig, L. Genzel, Optical-absorption of small metallic particles, Surf. Sci. 156 (1985) 678–700.

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