

The Antimicrobial Effects and Metabolomic Footprinting of Carboxyl-Capped Bismuth Nanoparticles Against *Helicobacter pylori*

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Abstract Organic salts of bismuth are currently used as antimicrobial agents against *Helicobacter pylori*. This study evaluated the antibacterial effect of elemental bismuth nanoparticles (Bi NPs) using a serial agar dilution method for the first time against different clinical isolates and a standard strain of *H. pylori*. The Bi NPs were biologically prepared and purified by a recently described method and subjected to further characterization by infrared spectroscopy and anti-*H. pylori* evaluation. Infrared spectroscopy results showed the presence of carboxyl functional groups on the surface of biogenic Bi NPs. These biogenic nanoparticles showed good antibacterial activity against all tested *H. pylori* strains. The resulting MICs varied between 60 and 100 µg/ml for clinical isolates of *H. pylori* and *H. pylori* (ATCC 26695). The antibacterial effect of bismuth ions was also tested against all test strains. The antimicrobial effect of Bi ions was lower than antimicrobial effect of bismuth in the form of elemental NPs. The effect of Bi NPs on metabolomic footprinting of *H. pylori* was further evaluated by ¹H NMR spectroscopy. Exposure of *H. pylori* to an inhibitory concentration of Bi NPs (100 µg/ml) led to release of some metabolites such as acetate, formic acid, glutamate, valine, glycine, and uracil from bacteria into their supernatant. These findings confirm

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that these nanoparticles interfere with Krebs cycle, nucleotide, and amino acid metabolism and shows anti-*H. pylori* activity.

Keywords Bismuth · *Helicobacter pylori* · Nanoparticle · Biosynthesis · Metabolomics

Introduction

Nanotechnology is currently a well-recognized term that describes novel and significantly improved physical, chemical, and biological properties of materials [1, 2]. Specifically, it describes the science field in which nanometer-scale materials are studied. These nanomaterials have drastically different characteristics from the bulk materials from which they are derived, which strongly depend on the size and shape of the substance [3]. Metal nanoparticles (NPs) are of particular interest due to their unique physiochemical and biological properties and are widely used in various applications [4–7].

Different metals such as silver, gold, and selenium have been chemically or biologically produced as nanoscale materials in recent years, and various reports on the interaction of these NPs with prokaryotic and eukaryotic systems are published in the literature [6–10]. Currently, organic salts of bismuth (i.e., bismuth subsalicylate, bismuth subcitrate, and bismuth subnitrate) are used as antimicrobial agents in medicine [11–15]. Bismuth preparations are particularly effective in the treatment of different gastrointestinal disorders including gastritis, duodenal ulcers, and traveler's diarrhea [14].

In the early 1980s, *Helicobacter pylori*, a small fastidious gram-negative bacilli, was isolated and an association between its infection and peptic ulcer disease was established.

Approximately 50 % of the world populations are known to be infected with *H. pylori*, mainly in the developing countries [15]. Eradication of this microorganism can result in curing of both infection and ulcer disease.

The management for the control of *H. pylori* infection involves the use of bismuth salts such as colloidal bismuth subcitrate, and proton pump inhibitors such as omeprazole, together with antibiotics like amoxicillin and metronidazole [16, 17]. Since the toxicity reported for elemental compounds at the nanoscale is lower than the toxicity of metal ion compounds, elemental NPs such as bismuth nanoparticles (Bi NPs) may be good candidates for replacement of other organic or inorganic salts of bismuth in future. The treatment failure rate for *H. pylori* infection is as high as 20 % and complete cure is not always achieved [16–18]. This failure is attributed to the selection for resistant strains of *H. pylori* due to repeated administration of anti-*H. pylori* medications, and the deep penetration of this bacterium into the gut epithelium, resulting in inaccessibility of antibiotics. These factors indicate a clear need for discovery and development of new anti-*H. pylori* agents [16–18].

In the present study, biogenic Bi NPs were intracellularly synthesized using a previously isolated bacterium [19]. The NPs were extracted from the bacteria, and their surface chemistry was studied by Fourier transform infrared spectroscopy (FTIR). Their antimicrobial effects were investigated by a serial agar dilution method against different clinical isolates of *H. pylori* as well as a standard test strain provided from American Type Culture Collection. Changes in intracellular metabolites in *H. pylori* following exposure to Bi NPs were also studied using nuclear magnetic resonance (NMR) metabolomic footprinting and the obtained NMR spectra were compared with control *H. pylori* cultures that were not treated with Bi NPs.

Materials and Methods

Biosynthesis of Bi NPs

Biosynthesis and purification of Bi NPs were conducted as described recently [19]. Briefly, nutrient broth medium containing 0.2 % bismuth subnitrate was prepared and inoculated with a pure-culture of *Serratia marcescens* isolated from the Caspian Sea (northern part of Iran). The medium was incubated on a rotary shaker at 150 rpm and 30 °C for 48 h. The bacterial cells were then removed by centrifugation and washed in 0.9 % NaCl to clean off any undesirable materials. The cells were disrupted by liquid nitrogen to release intracellular Bi NPs and an extracted using a two-phase system of 1:2 octanol and water. After 24 h, the released Bi NPs had settled in the bottom phase of the tubes. These settled materials were collected and re-extracted with chloroform. The upper phase was carefully transferred to a new test tube, centrifuged (10,000×g, 30 min), and sequentially washed with ethyl alcohol and distilled water. The sizes of the extracted Bi NPs before and after a long-term ultrasonic treatment (1 h) were determined by transmission electron microscopy (TEM). The composition of the prepared NPs was also studied by energy dispersive X-ray spectrometry (EDS).

Surface Chemistry of Biogenic Bismuth Nanoparticles

Functional groups present on the surface of Bi NPs prepared from *S. marcescens* were identified by drying the purified NPs and mixing them with potassium bromide. In the next step, a KBr tablet was made and subjected to FTIR. (Bruker Equinox55, Germany).

Antimicrobial Experiments

The *H. pylori* test strains used in this study were a standard strain (ATCC 26695) and four clinical isolates obtained from biopsies of the antrums of patients admitted to the endoscopy ward of Laleh Hospital in Tehran, Iran. The biopsies were transferred to the laboratory in thioglycolate broth medium at 4 °C within 5 h after removal from the patient. The samples were crushed and inoculated onto Columbia agar (Merck, Germany) plates supplemented with 10 % fetal bovine serum (FBS) (PAA, Austria) and 8 % defibrinated sheep blood, as well as an antibiotic supplement (vancomycin, polymyxin B, trimethoprim, cephalothin, amphotericin B) and campylobacter growth supplement purchased from Biolife, Italy. The plates were incubated at 37 °C under microaerophilic conditions with high humidity for 5 days [20, 21]. The strains were identified by colony morphology, gram staining, and oxidase, catalase, and urease tests.

The antimicrobial effects of biogenic Bi NPs against the selected test strains were determined by a modified serial solid agar dilution method adapted for *H. pylori* [21]. For this purpose, molten Columbia agar was subsequently supplemented with 10 % FBS, 8 % defibrinated sheep blood, and Bi NPs at concentrations of 40, 50, 60, 70, 80, 100, 120, and 140 µg/ml. Fresh cultures of each *H. pylori* test strain were harvested and suspended in normal saline to a final concentration of 10^8 CFU/ml (0.5 McFarland standard). The plates were inoculated with 5 µl of bacterial suspension, which provided an inoculum size of 10^5 – 10^6 CFU/spot [21]. Columbia agar media without any Bi NPs and Columbia agar supplemented Bi NPs without any inoculation were also used as positive and negative controls, respectively. All plates were incubated for 120 h in the microaerophilic condition described

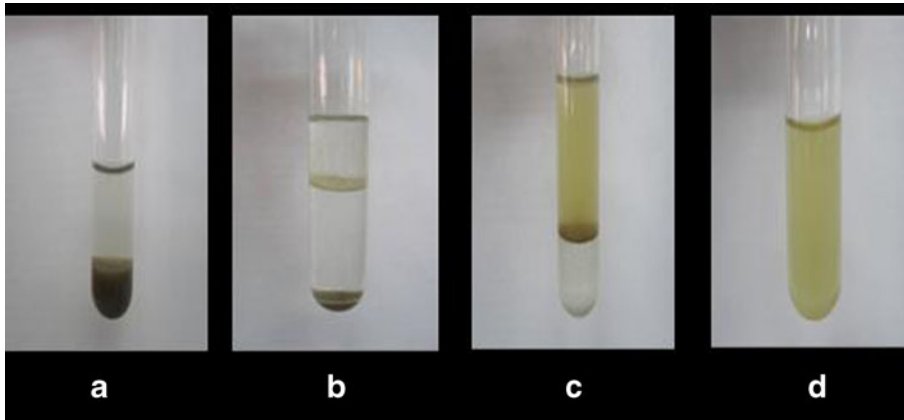


Fig. 1 Partitioning of Bi NPs in a two-phase aqueous–organic system. From *left to right*: *A* extraction of cell lysate by octanol (*upper phase*), *B* settled Bi NPs after octanol extraction, *C* re-extraction of collected Bi NPs by chloroform (*lower phase*), and *D* collected yellowish color colloid which contains pure Bi NPs

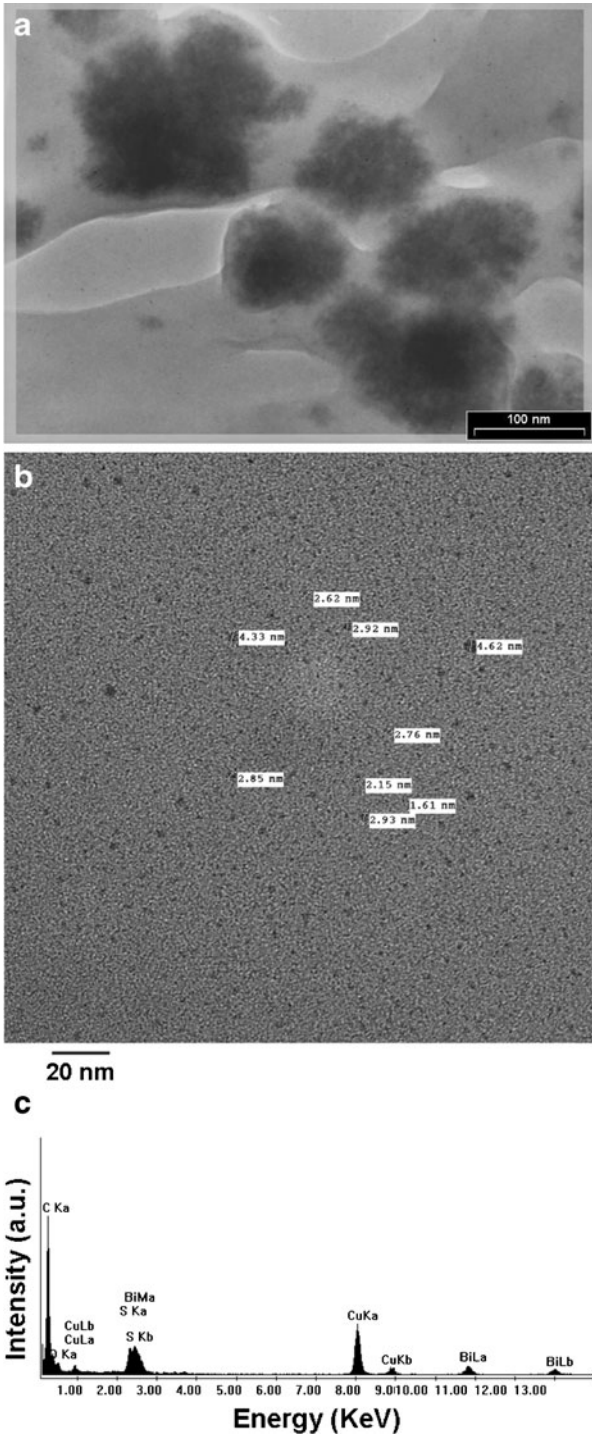
above. The MIC was determined as the lowest concentration of Bi NPs that inhibited bacterial growth. The antimicrobial tests were repeated twice.

Metabolomic Footprinting

^1H NMR footprinting of metabolite changes in the culture medium of *H. pylori* treated with Bi NPs was investigated using two selected test strains, the standard strain (ATCC 26695) and a clinical isolate. Test tubes containing 2 ml of *H. pylori* bacterial suspension in phosphate-buffered saline were prepared at the inoculum density of 10^8 CFU/ml (0.5 McFarland standard). Bi NPs were added to each tube to reach the concentration equivalent to the measured MIC (100 $\mu\text{g/ml}$). The suspension samples were agitated using magnets in microaerophilic conditions with high humidity at 37 °C for 24 h. After incubation, 0.1 % sodium azide was added to prevent sample contamination with other bacteria. The treated suspension samples were then centrifuged at $10,000\times g$ for 15 min and the supernatant was collected. The liquid supernatant was centrifuged again under the same conditions. The resulting upper phases were collected in glass vials and placed in an evaporator at temperatures not more than 40 °C to dry the samples. In the next step, aliquots (700 μl) of deuterium oxide containing 0.5 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid were added to the dried materials and thoroughly shaken. The samples were centrifuged at $10,000\times g$ for 15 min and the resulting clear liquids were subjected to NMR spectroscopy analysis.

The ^1H NMR spectra of the samples were obtained using a presaturation technique at 500 MHz on a Bruker Avance spectrometer equipped with a 5-mm broadband probe and operating in the Fourier transform mode. All chemical shifts were referred to DSS as internal standard and reported in parts per million. Metabolic products were identified by Chenomx software by comparison of chemical shifts that are representative of the structure of a particular molecule.

Fig. 2 TEM image of biogenic Bi NPs before (a) and after (b) long-term ultrasonic treatment. Lower illustration shows EDS of these Bi NPs which confirms the presence of Bi element. Additional peaks of copper and carbon elements are attributed to the grid used for TEM imaging



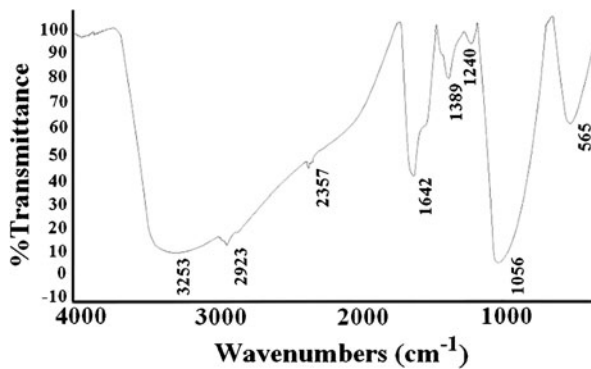


Fig. 3 Infrared spectrum of bismuth nanoparticles

Results and Discussion

Biosynthesis of Bi NPs and Their Surface Chemistry

In this investigation, *S. marcescens* was used to reduce Bi^{3+} to elemental Bi NPs. These biogenic Bi NPs are accumulated inside the bacteria and require release and purification for characterization and biological experiments. A recently described aqueous–organic two-phase partitioning method [19] can be successfully used for purification of elemental Bi NPs deposited within *S. marcescens*. Figure 1 shows the different steps of this extraction. A transparent yellowish colloid resulted, which contained the Bi NPs. Other undesirable materials were removed during the two-step extraction of cell lysates with octanol and chloroform.

TEM micrograph (Fig. 2a) shows that the small NPs formed larger aggregated NPs around (100 nm). These aggregated irregular-shaped NPs were further subjected to a long-term ultrasonic agitation (1 h) and studied again by TEM. TEM images at higher magnification (Fig. 2b) clearly shows very small NPs (≤ 5 nm), and no aggregation in the particles were observed after long-term ultrasonic treatment (1 h). Figure 2c depicts the EDS spectrum of non-aggregated NPs revealing the presence of Bi element peaks. These EDS peaks confirm the existence of Bi NPs. It should be mentioned that additional peaks, belonging to copper and carbon element, are attributed to the grid used for TEM imaging.

Table 1 MIC results for bismuth NPs against *H. pylori* strains

<i>H. pylori</i> test strains	Bismuth nanoparticle concentrations ($\mu\text{g/ml}$)								MIC $\mu\text{g/ml}$
	40	50	60	70	80	100	120	140	
No. 1	+ ^a	+	- ^b	-	-	-	-	-	60
No. 2	+	+	+	-	-	-	-	-	70
No. 3	+	+	+	-	-	-	-	-	70
No. 4	+	+	+	+	-	-	-	-	80
ATCC 26695	+	+	+	+	+	-	-	-	100

^a + indicates visible growth can be observed

^b - indicates no visible growth observed

The infrared spectrum of the purified Bi NPs is shown in Fig. 3. Two peaks appeared, with wave numbers of 1,056 and 1,642 cm^{-1} , which are attributed to individual group vibrations of carbonyl and —C—O— groups. A broad peak at 2,500–3,300 cm^{-1} associated with a hydroxyl group (—OH) could also be detected in the FTIR spectrum of purified Bi NPs (Fig. 3). This indicated that the biogenic Bi NPs carried a carboxylic acid functional group on their surfaces.

Antimicrobial Activity

Four clinical gram-negative *H. pylori* sp. with tiny transparent colonies were successfully isolated from biopsies of the antrums of patients and confirmed by positive oxidase, catalase, and strong urease activity. The antimicrobial activity of biogenic Bi NPs was evaluated in vitro against these four clinical isolates and a standard test strain of *H. pylori* (ATCC 26695). The Bi NPs showed good antimicrobial activity against all test strains of *H. pylori* (Table 1) The MIC of Bi NPs for the different *H. pylori* strains ranged from 60 to 100 $\mu\text{g/ml}$ (Table 1). The most sensitive sample was one clinical isolate of *H. pylori* with a MIC value of 60 $\mu\text{g/ml}$, whereas growth of three other clinical isolates of *H. pylori* was inhibited at the higher concentrations of Bi NPs (70 or 80 $\mu\text{g/ml}$). The highest MIC was observed for *H. pylori* (ATCC 26695) against biogenic Bi NPs (100 $\mu\text{g/ml}$).

In this investigation, the susceptibility of *H. pylori* (ATCC 26695) against bismuth subcitrate was also determined by a serial solid agar dilution method. No antibacterial effect was observed for bismuth in the form of bismuth subnitrate at concentrations lesser than 200 $\mu\text{g/ml}$ (corresponding to 280 $\mu\text{g/ml}$ of bismuth subnitrate). The antibacterial effect of bismuth in the form of elemental NPs against *H. pylori* was therefore higher than the anti-*H. pylori* effects of bismuth subnitrate, which is currently used as an antibacterial agent against this microorganism.

As mentioned in the introduction, a combination of bismuth organic salts and some antibiotics are currently used to manage *H. pylori* infections. The mechanism of action of bismuth compounds, despite their extensive therapeutic use, is not completely understood. It may cause cell wall degradation in *H. pylori* or may prevent bacterial adhesion on the gastric epithelium [22].

A recently published report in the literature indicates that Bi^{3+} ions inhibit the fumarase enzyme involved in the Krebs cycle. Fumarase is a key enzyme and modulates several oxidative reactions in the dicarboxylic acid arm of the Krebs cycle [23, 24]. The interaction of Bi^{3+} ions with this Krebs cycle enzyme, as well as its inhibitory effects on the other biological cycles involved in nucleotide pathways or urea metabolism, can lead to increases in free amino acids and other metabolites (i.e., uracil, formic acid, acetate) inside or outside of the bacterial cells [23, 24]. Therefore, analysis of small molecules or organic metabolites using footprinting techniques can provide important information about small changes in footprints of metabolic intermediates involved in different biochemical pathways.

The carboxyl-capped elemental Bi NPs biologically prepared during this investigation showed good antimicrobial activity against different test strains of *H. pylori* (Table 1). The effect of these Bi NPs on the footprints of various metabolites in culture media of *H. pylori* was studied by a ^1H NMR metabolite footprinting technique. Figure 4 shows the different ^1H NMR spectra recorded from the dried culture media inoculated with *H. pylori* (ATCC 26695) and incubated for 24 h in the absence (red lines) and presence of Bi NPs (blue lines). The ^1H NMR spectrum of the biogenic Bi NPs was also recorded and used as a control (green line). The major differences in chemical shifts of the three samples are indicated in Fig. 4. The appearance of signals corresponding to uracil (58.75 ppm) (NMR spectrum B), valine at 361 ppm (NMR spectrum C), and glycine at 355 ppm (NMR spectrum C) are clearly observed in *H. pylori* culture medium supplemented with Bi NPs (100 $\mu\text{g/ml}$). These substances were not found in

Fig. 4 ^1H NMR spectra recorded from dried culture media inoculated with *H. pylori* (ATCC 26695) and incubated for 24 h in the absence (*red lines*) and presence (*blue lines*) of Bi NPs. The *green line* indicates the ^1H NMR spectrum of the extracted Bi NP colloid. Spectra (**a–e**) show corresponding signals related to formic acid (**a**), uracil (**b**), valine and glycine (**c**), glutamate (**d**), and acetate (**e**). Whole spectra obtained during current metabolic footprinting analysis are also shown in image **f**

the other samples (untreated or control samples), and their presence may, therefore, be due to the effects of Bi NPs on metabolic pathways of *H. pylori*. Concentrations of other small molecules including formic acid (84 ppm) (NMR spectrum A), glutamate (24 ppm) (NMR spectrum D), and acetate (238 ppm) (NMR spectrum E) were also increased in the culture medium of *H. pylori* supplemented with Bi NPs. Illustration F in Fig. 4 shows a whole NMR spectrum recorded during this experiment. We obtained the same ^1H NMR metabolite footprinting results for the clinical isolate of *H. pylori* treated with Bi NPs, and no differences were observed between the metabolite footprinting pattern of standard *H. pylori* (ATCC 26695) and the clinical isolate (data not shown).

Conclusion

The *H. pylori* bacterium is a causative agent in gastric ulcer disease and gastric adenocarcinoma. Appearance of resistant test strains and insufficient patient compliance toward current anti *H. pylori* drugs are the main drawbacks of chemotherapy for *H. pylori*. Therefore, new therapeutic agents for curing this infection are constantly needed. In this study, elemental Bi NPs (<100 nm) were intercellularly synthesized by *S. marcescens*, extracted, and used for further experiments. The surface chemistry of these biogenic Bi NPs was studied by FTIR spectroscopy, which revealed the existence of carboxylic groups on the surface of the biogenic NPs prepared by *S. marcescens*. The antimicrobial activity of these carboxyl-capped Bi NPs was evaluated by a serial agar dilution method against different test strains of *H. pylori* and MIC values were calculated. Purified Bi NPs showed good antimicrobial activity against all tested bacterial strains. The effects of Bi NPs on *H. pylori* were also investigated by a ^1H NMR metabolite footprinting method. The concentrations of different small metabolites (some amino acids and organic acids) in the culture medium of *H. pylori* were increased in the presence of Bi NPs, indicating that these NPs interfere with vital biochemical pathways in *H. pylori*. Taken together, in vitro antimicrobial activity of Bi NPs against *H. pylori* was evident, so that these NPs may be useful for chemotherapy of *H. pylori* in the future.

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Conflict of Interest The authors declare that they have no conflict of interest in this paper.

References

1. Boisseau, P., & Loubaton, B. (2011). Nanomedicine, nanotechnology in medicine. *CR Physics*, 12, 620–636.
2. Roco, M. C. (2003). Nanotechnology: convergence with modern biology and medicine. *Current Opinion Biotechnology*, 14, 337–346.

3. Sahoo, S. K., Parveen, S., & Panda, J. J. (2007). The present and future of nanotechnology in human health care. *Nanomedicine: Nanotechnology, Biology, and Medicine*, 3, 20–31.
4. Arvizo, R. R., Bhattacharyya, S., Kudgus, R. A., Giri, K., Bhattacharya, R., & Mukherjee, P. (2012). Intrinsic therapeutic applications of noble metal nanoparticles: past, present, and future. *Chemical Society Reviews*, 41, 2943–2970.
5. Peng, H. I., & Miller, B. L. (2010). Recent advancements in optical DNA biosensors: exploiting the plasmonic effects of metal nanoparticles. *Analyst*, 136, 436–447.
6. Sau, T. K., Rogach, A. L., Jäckel, F., Klar, T. A., & Feldmann, J. (2010). Properties and applications of colloidal nonspherical noble metal nanoparticles. *Advanced Materials*, 22, 1805–1825.
7. Sokolov, K., Tam, J., Tam, J., Travis, K., Larson, T., Aaron, J., et al. (2009). Cancer imaging and therapy with metal nanoparticles. *Conf Proc IEEE Eng Med Biol Soc*, 2009, 2005–2007.
8. Dykman, L., & Khlebtsov, N. (2012). Gold nanoparticles in biomedical applications: recent advances and perspectives. *Chemical Society Reviews*, 41, 2256–2282.
9. Wang, D., Taylor, E. W., Wang, Y., Wan, X., & Zhang, J. (2012). Encapsulated nanoepigallocatechin-3-gallate and elemental selenium nanoparticles as paradigms for nanochemoprevention. *International Journal of Nanomedicine*, 7, 1711–1721.
10. Chen, R., Cheng, G., So, M. H., Wu, J., Lu, Z., Che, C. M., et al. (2010). Bismuth subcarbonate nanoparticles fabricated by water-in-oil microemulsion-assisted hydrothermal process exhibit anti-*Helicobacter pylori* properties. *Materials Research Bulletin*, 45, 654–658.
11. Chen, R., So, M. H., Yang, J., Deng, F., Che, C. M., & Sun, H. Z. (2006). Fabrication of bismuth subcarbonate nanotube arrays from bismuth citrate. *Chemical Communications*, 21, 2265–2267.
12. Sox, T. E., & Olson, C. A. (1998). Binding and killing of bacteria by bismuth subsalicylate. *Antimicrobial Agents and Chemotherapy*, 33, 2075–2082.
13. Stoltenberg, M., Martiny, M., Sorensen, K., Rungby, J., & Krogfelt, K. A. (2001). Histochemical tracing of bismuth in *Helicobacter pylori* after in vitro exposure to bismuth citrate. *Scandinavian Journal of Gastroenterology*, 2, 144–148.
14. Mahony, D. E., Lim-Morrison, S., Bryden, L., Faulkner, G., Hoffman, P. S., Agocs, L., et al. (1999). Antimicrobial activities of synthetic bismuth compounds against *Clostridium difficile*. *Antimicrobial Agents Chemother*, 43, 582–588.
15. Salih, B. A. (2009). Helicobacter pylori infection in developing countries: the burden for how long? *Saudi Journal of Gastroenterology*, 15, 201–207.
16. Andersen, L. P., Colding, H., & Kristiansen, J. E. (2000). Potentiation of the action of metronidazole on *Helicobacter pylori* by omeprazole and bismuth subcitrate. *International Journal of Antimicrobial Agents*, 14, 231–234.
17. Chuah, S. K., Tsay, F. W., Hsu, P. I., & Wu, D. C. (2011). A new look at anti-*Helicobacter pylori* therapy. *World Journal of Gastroenterology*, 17, 3971–3975.
18. O'Connor, A., Gisbert, J. P., McNamara, D., & O'Morain, C. (2011). Treatment of *Helicobacter pylori* infection 2011. *Helicobacter*, 16(Suppl 1), 53–58.
19. P. Nazari, M. A. Faramarzi, Z. Sepehrzadeh, M. R. Mofid, R. D. Bazaz, A. R. Shahverdi. Biosynthesis of bismuth nanoparticles using *Serratia marcescens* isolated from the Caspian Sea and their characterization. *IET Nanobiotechnology*, 6: 58–62.
20. McLaren, A. (1997). Methods in molecular medicine. In C. Clayton & H. Mobley (Eds.), *In vitro susceptibility testing of H. pylori* (Vol. 8, pp. 41–51). Totowa, NJ: Humana Press Inc.
21. European committee for antimicrobial susceptibility testing (EUCAST). (2000). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution. *Clinical Microbiology and Infection*, 6, 509–515.
22. Stratton, C. W., Warner, R., Coudron, P. E., & Lilly, N. (1999). Bismuth-mediated disruption of the glycocalyx-cell wall of *Helicobacter pylori*: ultrastructural evidence for a mechanism of action for bismuth salts. *The Journal of Antimicrobial Chemotherapy*, 43, 659–666.
23. Chen, Z., Zhou, Q., & Ge, R. (2012). Inhibition of fumarase by bismuth(III): implications for the tricarboxylic acid cycle as a potential target of bismuth drugs in *Helicobacter pylori*. *BioMetals*, 25, 95–102.
24. Ge, R., Sun, X., Gu, Q., Watt, R. M., Tanner, J. A., Wong, B. C., et al. (2007). A proteomic approach for the identification of bismuth binding proteins in *Helicobacter pylori*. *Journal of Biological Inorganic Chemistry*, 12, 831–842.