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Review

Biocoordination chemistry of bismuth: Recent advances

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Contents

Abstract

Bismuth compounds have been widely used in the clinic for centuries because of their high effectiveness and low toxicity in the treatment of a variety of microbial infections, including syphilis, diarrhea, gastritis and colitis. The efficacy of recently developed bismuth-based triple therapy in the eradication of *Helicobacter pylori* from patients exceeded the normal PPI-based (proton pump inhibitor-based) therapies. Apart from antimicrobial activity, bismuth compounds exhibit anticancer activities, ^{212}Bi and ^{213}Bi compounds have also been used as targeted radio-therapeutic agents for cancer treatment, and furthermore they have the ability to reduce the side-effects of cisplatin in cancer therapy. The investigation of bismuth interactions with potential targeting biomolecules, including peptides, proteins and enzymes will lead to an understanding of the mechanism of action of bismuth-containing complexes and in turn to the further application of bismuth in medicine.

Keywords: Bismuth; Cancer; Enzyme; Protein; Ulcer

1. Introduction

In contrast to the comprehensive database of other stable elements in the periodic table, bismuth perhaps has the least well established data bank, although bismuth has long been used in medicine. Bismuth was probably unknown to the

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Greeks and Romans, but it became familiar during the Middle Ages, notwithstanding its frequent confusion with other metals. From bismuth first name "wismut" given by Basil Valentine in 1450 to its latinized name "bisemutum", its elementary nature was imperfectly understood because of the impure specimens obtained by the early chemists [1]. These obscurities began to be finally cleared up with the research of Torbern Olof Bergman who reinvestigated bismuth properties and determined its reactions and for the first time accurately described this metal.

Nowadays, many bismuth compounds have been synthesized and their chemical properties were studied [2–6]. In this review, we summarize the recent progress in the bio-coordination chemistry of bismuth.

2. The chemistry of bismuth

Bismuth, a metallic chemical element with an atomic weight of 208.98038, was previously thought to be the heaviest stable element in the periodic table with the isotope $^{209}_{83}$Bi ($I = 9/2$) occurring naturally. The latest data showed that ^{209}Bi actually does decay but with an astonishingly long half-life of $1.9 (\pm 0.2) \times 10^{19}$ years [7]. Bismuth has two major oxidation states (Bi(III) with ionic radii of 1.03 and 1.17 Å for CN 6 and 8, respectively and Bi(V) with an ionic radius of 0.76 Å for CN 6), with the trivalent being the most common and stable form. Pentavalent bismuth is a powerful oxidant in aqueous solutions with a Bi(V)/Bi(III) potential of $E^{\circ} = 2.03$ V. Bi(III) readily hydrolyzes in aqueous solutions ($pK_a = 1.51$) and has a high affinity to both oxygen and nitrogen ligands [8,9]. However, it prefers to coordinate to thiolate groups (–SR) when thiol-containing ligands such as cysteine and glutathione are available [10–12]. The coordination chemistry of bismuth complexes with aminopolycarboxylate and polyaminopolycarboxylate ligands has been summarized recently [9]. Corresponding to its various coordination numbers from 3 to 10 [13,14], the structures of Bi(III) compounds are irregular, from pyramidal (CN: 3), octahedral (CN: 6, $[Bi_6O_4OH)_4]^{6+}$) [15], bicapped trigonal prism (CN: 8, $[Bi(HEDTA)]\cdot 2H_2O$) [16] and square antiprism [17,18] to tricapped trigonal prism (CN: 9, $[Bi(OH_2)_9](CF_3SO_3)_3$) [19].

Most of the Bi(V) complexes are five-coordinated [20], although Bi(V) in triarylbismuthate complexes such as $Ar_3Bi(HCO_2)_2$ (where Ar = Ph, *p*-Tol) [21], are six-coordinated. Almost all of Bi(V) complexes are very unstable in aqueous media except a recently reported seven-coordinated Bi(V) tropolonato complex, tri(aryl)tropolonatobismuth(V). The stability of this Bi(V) complex may be attributed to the steric shielding of the bismuth ion [22]. In some Bi(V) complexes such as $BiR_3(O_2CR')_2$, synthesized *via* the reaction of BiR_3Cl_2 with $Ag(O_2CR')$, the Bi(V) centre shows interesting unusual stereoselectivity towards the chiral ligand $R^*CO_2^-$. $BiR_3(O_2CR')_2$ can be used as building units for the assembly of extended structures *via* hydrogen-bonding [20].

3. Bismuth in medicine

The use of bismuth compounds in medicine can be traced back to the Middle Ages, as the first account of using bismuth as medicines was in reported in 1786 by Louis Odier for the treatment of dyspepsia [23]. Nowadays, based on the gradually understood characteristics of this element, many bismuth compounds have been synthesized and some have clinical and health applications. Currently, the major medicinal use of bismuth compounds is focussed in two fields: antimicrobial and anticancer.

3.1. Bismuth as an antimicrobial agent

In the antimicrobial realm, bismuth compounds have been used in the treatment of various microbial infections such as syphilis (sodium/potassium bismuth tartrate, bismuth quinine iodide, iodobismitol, bismuth chloride, etc.), colitis (bismuth subnitrate, bismuth citrate), wound infection (bismuth oxide), quartan malaria (sodium bismuth thioglycolate), dyspepsia (bismuth subsalicylate, bismuth subnitrate, etc.), diarrhea (bismuth subsalicylate, bismuth nitrate, etc.) and peptic ulcers (colloidal bismuth subcitrate, bismuth citrate, bismuth subnitrate, etc.) [24,25]. Three bismuth compounds, bismuth subsalicylate (BSS, Pepto-Bismol®; the Procter & Gamble Company, Cincinnati, Ohio, USA), colloidal bismuth subcitrate (CBS, De-Nol®; Gist Brocades and Yamanouchi), and ranitidine bismuth citrate (RBC, Tritec® and Pylorid®, GSK) are used worldwide to treat various gastrointestinal diseases which are related to the infection of *Helicobacter pylori* (*H. pylori*, an organism which was first discovered in 1983). A new complex of bismuth with *d*-polygalacturonic acid, the so-called "colloidal bismuth pectin", was approved in clinical use in China for the treatment of peptic ulcers [26]. To completely eradicate *H. pylori* infections, bismuth-based triple or quadruple regimens, which combine the bismuth drugs with antibiotics such as amoxicillin, clarithromycin or nitroimidazole, are often used. Compared to the normally used PPI-based (proton pump inhibitor-based) triple therapies, bismuth-based triple regimens are more efficient in patients' first, second and third line *H. pylori* eradication therapies [27]. As shown in Fig. 1, the efficacy of bismuth-based triple therapies exceeded PPI-based therapies in the first and second

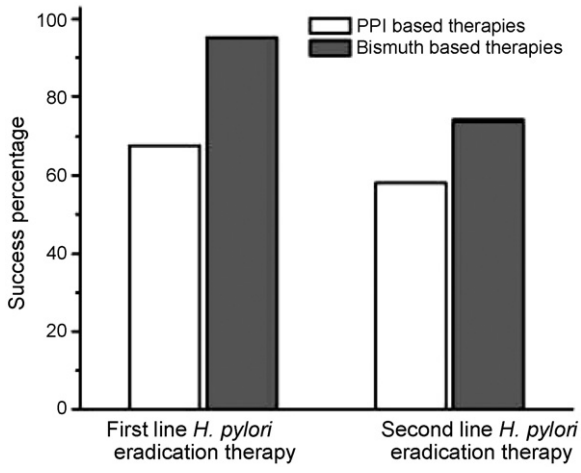
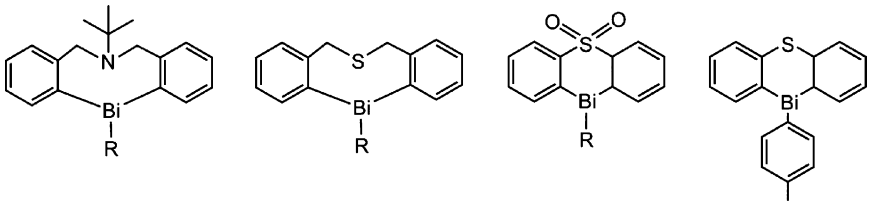


Fig. 1. The efficacies of PPI-based (white) and bismuth-based (grey) regimens to *H. pylori* first and second line eradication.



Scheme 1. Structures of the cyclic organobismuth compounds **1–8** [30].

line *H. pylori* eradication therapies, suggesting that these kinds of regimens should be used more widely in the clinic.

In addition to the currently used bismuth compounds, the development of new bismuth-based compounds may provide some promising antimicrobial agents. The antibacterial properties of bismuth are greatly enhanced when bismuth is combined with certain lipophilic thiol compounds [28]. Comparing to $Bi(NO_3)_3$, the antimicrobial activity was enhanced by 25–300-fold when combined the $Bi(NO_3)_3$ with seven different thiols (1,3-propanedithiol, dimercaprol (BAL), dithiothreitol, 3-mercapto-2-butanol, β-mercaptoethanol, 1-monothioglycerol, and mercaptoethylamine) [28]. Among them, bismuth dimercaprol (BiBAL) was active against a broad range of bacteria, particularly active against *H. pylori* (MIC, 2.2 μM), *Staphylococcus aureus* (*S. aureus*, MIC, 7.5 μM), and *C. difficile* (MIC, 7.5 μM) and was least active against the enterococci (MIC, 63 μM) and certain anaerobes (MIC, 15–100 μM) [28]. Recently, the inhibition effect of bismuth-dithiol against rho, an enzyme which is essential in many Gram-negative organisms, was reported [29]. The BiBAL solution (Bi:BAL = 3:1) acted as a noncompetitive inhibitor with respect to ATP in the rho poly(C)-dependent ATPase with IC_{50} value of 60 μM, and as a competitive inhibitor with respect to ribo$(C)_{10}$ in the poly(dC)-ribo$(C)_{10}$-dependent ATPase assay [29].

A series of cyclic organobismuth compounds exhibited potential antimicrobial activities (Scheme 1) [30,31]. The eight-membered-ring bismuth compounds (compounds **1–3** except **4**) were more active than the six-membered ones (compounds **5–8**). The MIC values of bismuth compounds **1–3** are less than 1 μM (0.50, 0.91, and 0.55 μM, respectively) against *S. aureus*, while the MIC values for compounds **5–8** are in the range of 7–34 μM (34.73, 7.75, 8.84 and 33.03 μM, respectively) [30]. The antifungal activities of organobismuth(III) and (V) compounds are governed by the facility of nucleophilic reaction at the metal center and the Lewis acidic bismuth center is an active site [31].

In recent years, nanomaterials have been widely used as medicines in many fields [32], including antibacterial [33,34], drug delivery [35,36], pathogen detection [37], tumor destruction [38] and MRI contrast enhancement [39]. Bismuth subcarbonate ($(BiO)_2CO_3$) nanotube was synthesized and characterized using bismuth citrate as a template [40]. The TEM images showed that two $(BiO)_2CO_3$ nanotubes are lying side by side with nearly identical diameters (4.2 and 4.3 nm) (Fig. 2a). The fringe spacing is about 0.278 nm, which is very close to the interplanar spacing of the (1 1 0) lattice planes of $(BiO)_2CO_3$ (Fig. 2b). Importantly, these nanotubes showed slightly enhanced activities (10 μg/mL) against *H. pylori* compared with the clinically used antiulcer drug, colloidal bismuth subcitrate (~20 μg/mL) under identical conditions. These nanotubes could be used as a "capsule" in bismuth triple and quadruple therapeutic approaches for *H. pylori* infection, or as a "carrier" for other drugs.

3.2. Bismuth as an anticancer agent

^{212}Bi ($t_{1/2}$ = 61 min) and ^{213}Bi ($t_{1/2}$ = 46 min) are α-particles emitting radionuclides with linear energy transfer, and are much more potent than β-particles such as ^{90}Y [41,42]. Both of them have a series of branched decay, resulting in the emission of α-/β-particles and γ-rays (Scheme 2). Similar to other α-particles,

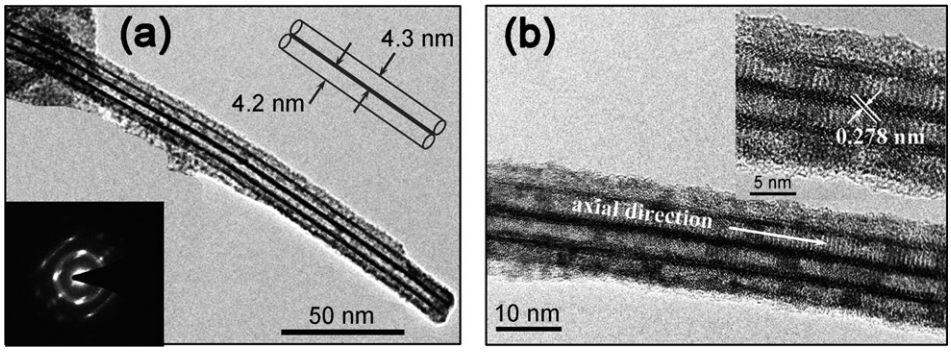
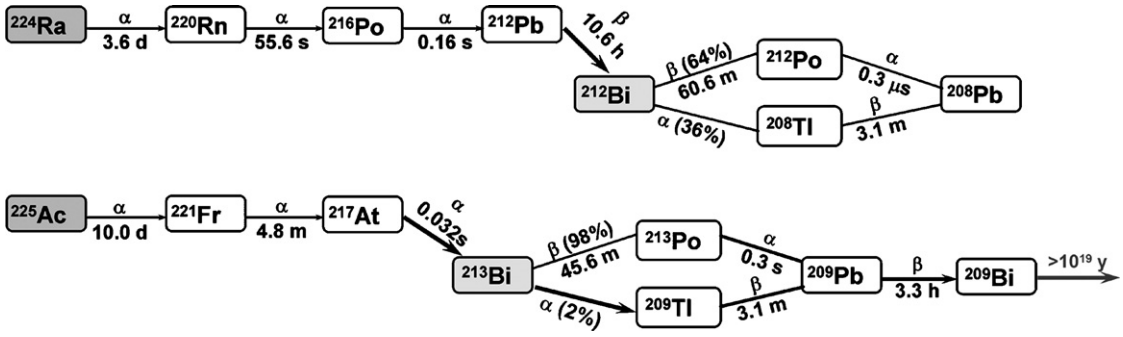


Fig. 2. TEM images of a bundle of $(BiO)_2CO_3$ nanotubes and its corresponding SAED pattern (a). The *d*-spacing between the lattice planes is 0.278 nm, corresponding to the (1 1 0) plane (b) (adapted with permission from Ref. [40]).



Scheme 2. ^{212}Bi and ^{213}Bi decay scheme.

^{212}Bi and ^{213}Bi have a short ranged penetration (50–80 μm), which could potentially reduce nonspecific irradiation of normal tissues around the target cells. Recent investigations have shown promising potentials of ^{213}Bi as a novel therapeutic agent for the small volume tumors. The relatively short half-lives of ^{212}Bi and ^{213}Bi require rapid dose delivery, and at the same time rapid attachment of the metal to carrier molecules. They can be generated from ^{224}Ra and ^{225}Ac generators (Scheme 2), respectively [43–45] and a ^{213}Bi generator, providing up to 25 mCi of pure and chemically reactive ^{213}Bi, has been developed for clinical use [46,47].

To conduct bismuth to the site of diseases effectively, a chelate ligand such as aminopolycarboxylate or polyaminopolycarboxylate ligand (iminodiacetate, nitrilotriacetate, pyridinedicarboxylate, diethylenetriaminepentaacetate (DTPA), 1,4,7,10-tetra-azacylododecane 1,4,7,10-tetraacetate (DOTA) and porphyrins) [41,48], is always used to form a stable complex with the radionuclide. More frequently, the strong chelate ligand is conjugated to a monoclonal antibody (mAb) or a fusion protein, a standard treatment for tumors, *via* modification of the ligand to produce a bismuth-radiolabeled "complex" eventually. In this way the metal radiolabeled "complex", once introduced into the host, targets specific cell types and sites of diseases [44,45,49,50]. It releases the α-particles only at or in the tumor tissues, thereby minimizing its damage to the surrounding normal tissues.

The conjugates of ^{213}Bi CHX-A-DTPA complex with a humanized anti-CD33 antibody HuM195 [51], an anti-CD45 mAb [52] and an anti-prostate-specific membrane antigen antibody (J591) [53,54] have been used in preclinical models of leukemia and prostate cancer, respectively [46,55]. Sequential therapy with cytarabine and ^{213}Bi-labelled HuM195 was entered in a phase I/II clinical trial for the treatment of advanced myeloid leukaemia [56]. Clinical trial using ^{213}Bi-labeled HuM195 demonstrated specific and potent cell killing ability against leukemia with no significant toxicity [51] and showed that targeted α-particle therapy is feasible in humans. ^{213}Bi-C595 can effectively inhibit growth of pancreatic cell clusters and pre-angiogenic lesions *in vivo*, indicating that ^{213}Bi treatment may have a role as adjuvant therapy to prevent early recurrence [57].

To overcome the short half-lives of the radionuclides and reduce radiation doses to normal organs, and improve tumor-to-normal organ dose ratios, a pre-targeted approach of radioimmunotherapy has been developed. One pre-targeting method involves administration of a monoclonal antibody or engineered targeting molecule conjugated to streptavidin, followed by administration of a biotinylated *N*-acetylgalactosamine-containing "clearing agent" to remove excess circulating antibody [58,59]. This method takes advantage of the extremely high affinity of avidin-biotin binding and rapid pharmacokinetics of the small molecule biotin. Pre-targeting of tumor masses with streptavidin results in rapid and high concentration of the ^{213}Bi-DOTA-biotin conjugate in tumors, leading to tumor responses, and to complete elimination of tumor xenografts in some mice [59]. Careful evaluation of the dose delivered to the kidney *in vivo* and a strategy of slow escalation of the dose may allow clinical use in the future.

Anticancer activities of a series of bismuth dithiocarbanates and tropolones complexes were examined recently and the mode of actions was suggested to be related to metabolic stress and cell survival, i.e., inhibition of enzymes [60]. Early studies showed that organometallic bismuth compounds exhibit anticancer activities such as $\{Na_2[BiO(mp)_3]\cdot H_2O\}$ (mp = 6-mercaptopurine) [61], $\{[Bi(tgn)_3(H_2O)]\cdot 3.5H_2O\}$ (tgn = thioguanine) [62], bismuth thiolates [63] and arylbismuth oxine complexes [64]. As shown in Table 1, $[CH_3Bi(p\text{-}SC_6H_4NH_2CH_3)_2]^{2+}2I^-$ exhibited the best antitumor activity to fluid Ehrlich ascites tumor with the therapeutic index (TI) value of 5.0, while the compounds $CH_3Bi(SCH_3)_2$ and $CH_3Bi(p\text{-}SC_6H_4NH_2)_2$ have lower therapeutic indices, with TI

Table 1
Pharmacological data of bismuth thiolates against fluid Ehrlich ascites tumor [63]

Compound	Optimum doses (μM/g)	LD_{50} (μM/g)	LD_{100} (μM/g)	T.I.[a]
$CH_3Bi(SCH_3)_2$	0.05–0.06	0.1	0.25	3.2
$CH_3Bi(p\text{-}SC_6H_4NH_2)_2$	0.08	0.18	0.30	4.3
$[CH_3Bi(p\text{-}SC_6H_4NH_2CH_3)_2]^{2+}2I^-$	0.08–0.11	0.13	0.21	5.0

T.I.: therapeutic index, determined by calculation LD_{50}/ED_{75}.

Table 2
Growth inhibition of arylbismuth oxine complexes against L1210, L1210/DDP and SKOV-3/*in vitro* [64]

Compound	L1210	IC_{50} (μM) L1210/DDP[a]	SKOV-3
H(OX)[b]	4.1	0.77	6.8
H(MeOX)	8.3	13	85
$Bi(OX)_3$	0.26	0.33	0.29
$Bi(OX)_2I$	0.25	0.22	0.77
$PhBi(OX)_2{\cdot}EtOH$	0.19	0.28	0.75
$PhBi(MeOX)_2$	0.56	0.37	2.9
$[NaPhBi(OX)_3]$	0.34	0.12	0.64
$[KPhBi(OX)_3]$	0.35	0.27	0.66
$Bi(OH)_3$	>5	>5	n.d.
Ph_3Bi	42	n.d.	n.d.
Ph_2BiI	0.29	0.31	n.d.
$PhBiI_2$	1.3	1.1	n.d.
Cisplatin	0.6	6.7	3.1

[a] Corresponding to cisplatin resistant line L1210/DDP.
[b] H(OX) = quinolin-8-ol.

values of 3.2 and 4.3, respectively [63]. The anticancer activities of arylbismuth oxine complexes against L1210, L1210/DDP and SKOV-3 are shown in Table 2. Although the free ligands of H(OX) and H(MeOX) exhibit the activity to all three types of tumors, their bismuth complexes exhibit much higher activities, indicative of an enhancing role of the metal. The enhanced activitites are probably due to the enhancing delivery of active H(OX) or H(MeOX) by bismuth [64]. Recently, a water-soluble bismuth complex, Bi-TPC (TPC = 1,4,7,10-tetrakis(2-pyridylmethyl)-1,4,7,10-tetraazacyclododecane), exhibited a very high cytotoxic activity against melanoma B16–BL6 cells with an inhibition rate of 82% at a concentration of 0.25 μM after 48 h. The IC_{50} of the complex is 41 nM, which is around 100 times more potent than cisplatin (*cis*-diamminedichloroplatinum, *cis*-$Pt(NH_3)_2Cl_2$, CDDP). The complex binds to DNA under physiologically relevant conditions probably *via* a non-covalent interaction [65].

Application of bismuth compounds in chemotherapy also lies in their ability to reduce the side-effects of anticancer drugs such as cisplatin without affecting the anticancer activity of the drug. Cisplatin and its analogues are widely used anticancer agents and have been used in medicine to treat cancer patients in the clinic. However, the side effects of CDDP lower the efficacy and narrow the utilization of CDDP in cancer therapy [66–69]. Recently, bismuth subnitrate (BSN) was found to be able to reduce the side-effects of cisplatin [70,71]. Interestingly, citrate was also used with BSN as a transporter to enhance the bismuth distribution in kidneys. The high absorption of bismuth by kidneys can increase the amount of renal metallothionein (MT), a cysteine-rich small protein, which plays a significant role in protection against the toxicity of heavy metals, alkylating agents and free radicals [72–76]. BSN-citrate as a MT inductor can increase the MT concentration in kidneys by about 30-fold comparing to the use of BSN alone, and did not cause significant increase in tumor MT concentrations. The pretreatment of BSN-citrate can reduce the blood urea nitrogen (BUN) level that was determined as an indicator of renal toxicity (Fig. 3). Recent microarray analysis demonstrated that metallothionein gene in microphage cells

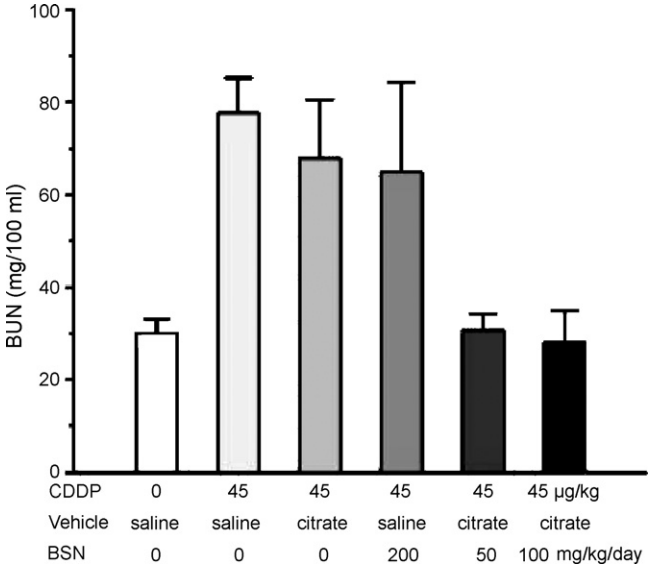


Fig. 3. Effect of orally pretreatment of bismuth subnitrate (BSN) with saline or citrate buffer on renal toxicity of cisplatin (CDDP) in mice inoculated with Meth-A fibrosarcoma. Blood urea nitrogen (BUN) values were measured 5 days after the CDDP injection [71].

increased by at least 10-fold upon exposure of 50 μM bismuth citrate [77].

4. Structure modeling of bismuth drugs

4.1. Bismuth subsalicylate

Bismuth subsalicylate (BSS) is one of bismuth drugs in the treatment of a variety of gastrointestinal ailments including duodenal and peptic ulcers, ulcerative colitis, and diarrhea [25,78–82]. Considerable efforts have been made to model the structure of BSS through bismuth salicylate $[Bi(Hsal)_3]$ (H_2sal = (2-HO)C_6H_4COOH) "trapped" by chelating amines, e.g., $[Bi(Hsal)_3(bpy)]_2{\cdot}(C_7H_8)_2$, (bpy = 2,2′-bipyridine) [83], bismuth thiosalicylate complexes [84], and the structurally uncharacterized $[Bi(Hsal)_3]$ compound [85,86]. Recently, a new structure model of BSS through two bismuth oxosalicylate clusters, $[Bi_{38}O_{44}(Hsal)_{26}(Me_2CO)_{16}(H_2O)_2]{\cdot}(Me_2CO)_4$ and $[Bi_9O_7(Hsal)_{13}(Me_2CO)_5]{\cdot}(Me_2CO)_{1.5}$ was reported and the mechanism of the hydrolysis and core formation were proposed [87]. The X-ray diffraction study on a variety of crystals from several reaction mixtures indicates that the crystal of $[Bi_9O_7(Hsal)_{13}(Me_2CO)_5]$ initially predominates with co-crystallization of a relatively small amount of the large cluster $[Bi_{38}O_{44}(Hsal)_{26}(Me_2CO)_{16}(H_2O)_2]$, which has 38 bismuth atoms. However, the transposition of predominance of the two types of crystals when extended the crystal growth time indicates that the $[Bi_{38}O_{44}(Hsal)_{26}(Me_2CO)_{16}(H_2O)_2]$ is the least soluble and most thermodynamically stable form. The structural building blocks for these two clusters are six octahedral Bi atoms with eight O atoms $[Bi_6O_8]^{2+}$, a common building block of bismuth-oxo compounds [4] and the core of $[Bi_9O_7(Hsal)_{13}(Me_2CO)_5]$ lies at the heart of $[Bi_{38}O_{44}(Hsal)_{26}(Me_2CO)_{16}(H_2O)_2]$ (Fig. 4). This reveals a possible process for hydrolysis and core forma-

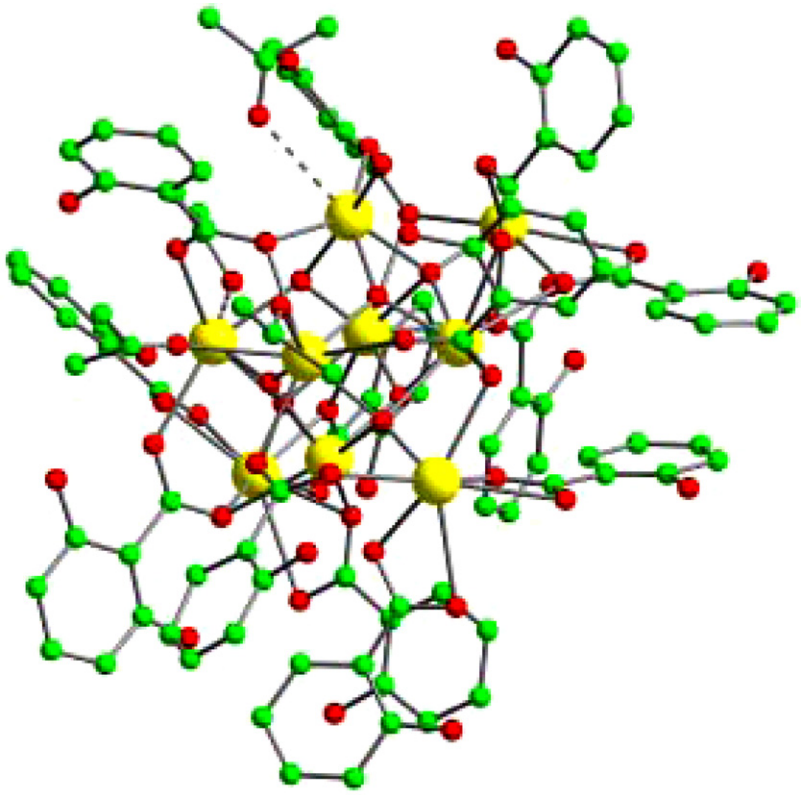


Fig. 4. Crystal structure of $[Bi_9O_7(Hsal)_{13}(Me_2CO)_5]$. Color code: Bi, yellow; O, red; C, green [87].

tion, and indicates that $[Bi_9O_7(Hsal)_{13}(Me_2CO)_5]$ gives rise to the complex $[Bi_{38}O_{44}(Hsal)_{26}(Me_2CO)_{16}(H_2O)_2]$.

4.2. Colloidal bismuth subcitrate

Among the modern bismuth-containing pharmaceuticals, colloidal bismuth subcitrate (CBS, De-Nol® and Lizhudele®) is one of the most widely used bismuth drugs in many countries [88]. The function of CBS possibly involves the formation of bismuth citrate "polymeric coating" on ulcer craters to prevent the erosion by gastric acid [89,90]. The empirical formula of CBS was previously often given as: $K_3(NH_4)_2[Bi_6O_3(OH)_5(Hcit)_4]$ [91], but has been changed to "polymeric bismuth citrate complex" in the latest edition to reflect its complicated polymeric features [92]. Nine different bismuth citrate adducts have been isolated under different conditions and characterized by X-ray crystallography and NMR spectroscopy. Most of them contain the stable $[Bi(cit)_2Bi]^{2-}$ dimer with additional O^{2-}, OH^- and H_2O ligands. This dimeric unit can further aggregate *via* citrate bridging to form channels and sheets *via* hydrogen-bonding, which probably accounts for the high solubility of CBS. Efforts have been made to elucidate the structure of CBS, but the bismuth citrate complexes have complicated structures, depending on various conditions. Recently, a structure of bismuth citrate complex crystallized at pH 3 to mimic stomach condition was solved. Three types of bismuth citrate $[Bi(cit)_2Bi]^{2-}$ dimers were noticed [93]. These dimers serve as building blocks and further assemble into a two-dimensional sheet and three-dimensional polymer (Fig. 5) [93]. This may reveal a possible process that CBS rearranges from colloidal particles such as $[Bi_6O_4(cit)_4]^{6-}$ and $[Bi_{12}O_8(cit)_8]^{12-}$ at a neutral pH value [94], to sheets and 3D polymers under acidic condition (e.g., in the stomach) due to a rapid ligand exchange as demonstrated by NMR spectroscopy [93]. The presence of the channels (ca. 5×10 Å^2) in the bismuth citrate polymer may allow other molecules such as ranitidine to diffuse into.

5. Bismuth binding biomolecules

5.1. Bismuth interacting peptides

Both cysteine and the tripeptide glutathione (γ-L-Glu-L-Cys-Gly, GSH) can prevent the precipitation of CBS at pH 2. Glutathione is present in many cells at relatively high concentrations (ca. 0.5–10 mM) and may play a role in the transport of Bi(III) in cells and biofluids. Electrospray mass spectrometry (ESI-MS) of bismuth complexed to various cellular biomolecules such as cysteine and GSH showed that various molecular species of Bi(III) with both cysteine and GSH corresponding a stoichiometry of Bi:GSH (or cysteine) = 1:1,1:2 and 1:3 in the gas phase (Fig. 6), were identified [11,12], which provides the basis for determining the molecular speciation of bismuth *in vivo*. The structures of mono- and bis-complexes were proposed and the thermal and hydrolytic stability of the Bi–S bond may act as an anchor for these ligands, indicative of a primary biochemical role of bismuth drugs. Ternary complexes of bismuth with cysteine and either citrate or 1,10-phenanthroline (phen) may form and the X-ray structure

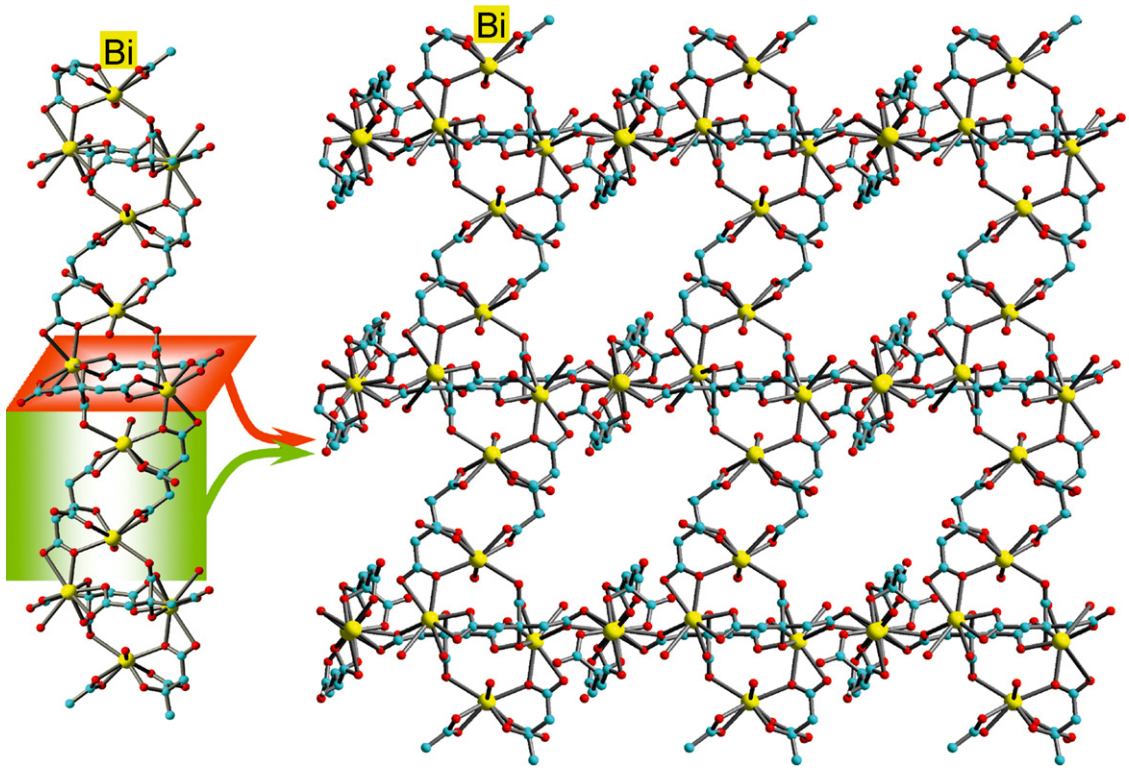


Fig. 5. X-ray polyanionic structure of $[Bi(cit)_2Bi]_n^{2n-}$. The crystal lattice was aggregated by three different dimers along the *c* axis (dimers **I** and **II**) and *a*-axis (dimers **II** and **III**). Color code: C, cyan; O, red; Bi, yellow (adapted with permission from Ref. [93]).

demonstrated that cysteine coordinates to the metal center in a bidentate mode *via* a thiolate sulfur and an oxygen [95,96].

NMR studies have shown that Bi(III) coordinates to the thiolate sulfur of GSH to form a $Bi(GS)_3$ complex, and the metal ions appear to pass through red blood cell membrane slowly and forms an intracellular complex with GSH [10]. The transport of each Bi(III) ion results in the co-transport of three glutathiones [97]. Antimicrobial activities of bismuth thiolate complexes are probably due to enhanced intracellular bismuth uptake facilitated by thiolate ligands.

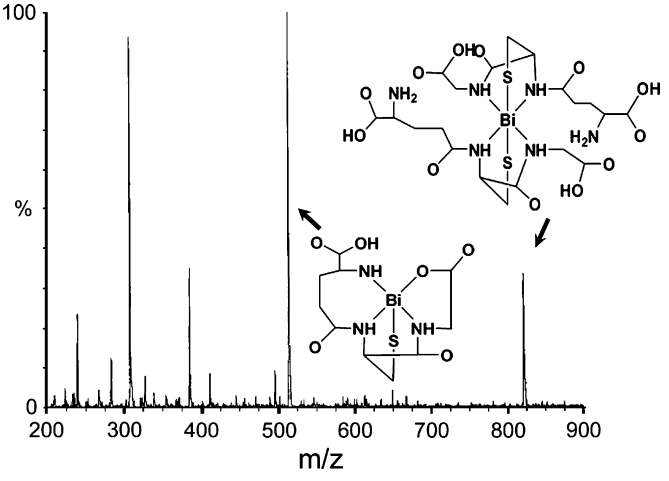


Fig. 6. ESI-MS of a solution containing $Bi(NO_3)_3$ and GSH. Peaks at *m/z* 514 and 821 are assigned to $[Bi(GSH)\text{-}4H]^-$ and $[Bi(GSH)_2\text{-}4H]^-$ (adapted with permission from Ref. [11]).

Gastrin is a small peptide that can stimulate the growth of *H. pylori* directly [98,99]. A recent study shows that bismuth may inhibit the biological activity of glycine-extended gastrin 17 (Ggly) [100]. Both glycine-extended and amidated gastrin 17 bind two Fe(III) or Bi(III), and the metal probably coordinates to the side-chain of Glu-7. Bismuth can inhibit the activity of glycine-extended gastrin 17 to stimulate inositol phosphate production, cell proliferation and cell migration.

5.2. Bismuth binding proteins

5.2.1. Transferrin and lactoferrin

Transferrin is an 80 kDa glycoprotein which transports Fe(III) in blood, and is recognized by cell surface receptors when fully loaded with Fe(III) in its two binding sites. Moreover, the affinity of metal-loaded transferrin for the transferrin receptor is much higher than that of apo-transferrin. The diferric protein is internalized by cells, placed in vesicles (endosomes) where the pH is lowered to 5.5 and the Fe(III) is released [101,102]. Since it is only 30% saturated with Fe(III), the protein is therefore regarded a "carrier" for metal ions and metallodrugs [103]. About 70% bismuth was found to bind to transferrin even in the presence of a large excess of albumin (albumin:transferrin = 13:1) at pH 7.4, 10 mM bicarbonate [104]. Bismuth(III) binds strongly to both the C- and N-lobe iron binding sites of human serum transferrin and recombinant N-lobe transferrin [105,106]. The uptake of Bi(III) by apo-transferrin from bismuth citrate is slower (h) than that of bismuth nitrilotriacetate (Bi(NTA)) (min). The bound carbonate (CO_3^{2-}) as a synergistic anion for bismuth binding

was approved by ^{13}C NMR and it cannot readily be removed even after extensive dialysis. Binding of bismuth occurs preferentially to the C-lobe of transferrin. The order of lobe-loading has been confirmed by 2D NMR studies using recombinant ε-[^{13}C]Met-hTF [107]. The Bi(III)-induced changes in shifts of the $^{1}H/^{13}C$ resonances of the labeled transferrin are similar to those induced by Fe(III), Al(III) and Ga(III), indicative of similar conformational changes upon binding. Recent X-ray structure of aluminium-bound ovotransferrin (Al_2-oTF) revealed that both the overall organization of the Al-bound form and the two metal-binding sites (four residues from the protein, i.e., one Asp, two Tyr and one His, and one bicarbonate anion) are almost the same as the iron-bound form [108]. The extent and mode of the closure of the Al-bound form are almost the same as in the diferric form, supporting the participation of transferrin in the transport of Al(III) ions *in vivo via* the receptor mediated process.

The detailed kinetics of Bi(III) translocation between Bi(NTA) and human serum transferrin were examined [109]. Uptake of the metal by transferrin is a multi-step kinetic process. The first step is the rapid metal exchange (ms) between Bi(NTA) and the C-site of apo-protein in interaction with bicarbonate, followed by a proton loss (pK_{1a} of 8.6) to yield a kinetic intermediate. The intermediate subsequently undergoes a conformational change. The final step, also the rate-determining step, is the uptake of Bi(III) by the N-site. Interaction of the Bi-loaded transferrin with transferrin receptor (TFR) follows a single step-process, in contrast to a two-step process of Fe_2-hTF and TFR. The binding affinity between Bi-loaded transferrin and TFR was determined to be 4 μM, around three orders of magnitude lower than that of Fe_2-hTF and TFR (23 nM). Bismuth-loaded transferrin probably binds to the transferrin receptor at the helical domain of the protein, which is specific for the C-site of transferrin [102].

Lactoferrin, widely found in a variety of secretory fluids, such as milk, bile, pancreatic juice, and small intestinal secretions (mucosal fluid) [103,110,111], is another major iron-binding protein in the transferrin family. Lactoferrin binds Fe(III) more tightly with an affinity about 100 times higher than that of human transferrin [112], and it has been speculated that lactoferrin has a bacteriostatic function in depriving microorganisms of essential iron required for their growth [113]. Bismuth binds to human lactoferrin at the specific iron sites together with carbonate or oxalate as the synergistic anion (Fig. 7). Similar to iron binding to the protein, lactoferrin binds Bi(III) strongly and at the same time reversibly [114]. More importantly, the Bi_2-hLF complex can compete with the $^{59}Fe_2$-hLF complex for both membrane (Fig. 8a) and intracellular (Fig. 8b) binding, almost as effectively as the Fe_2-hLF and Ga_2-hLF complexes. The apo-hLF is also as effective as metal-loaded lactoferrin in competing with the $^{59}Fe_2$-hLF complex, which is markedly different from the transferrin. These are in agreement with the results describing the recognition of lactoferrin by the parasite *Leishmania donovani* [115,116] which shows that lactoferrin binding is independent of whether the protein is metal-loaded [115]. All the results revealed that the lactoferrin receptor is more promiscuous than the transferrin receptor of rat IEC-6 cells.

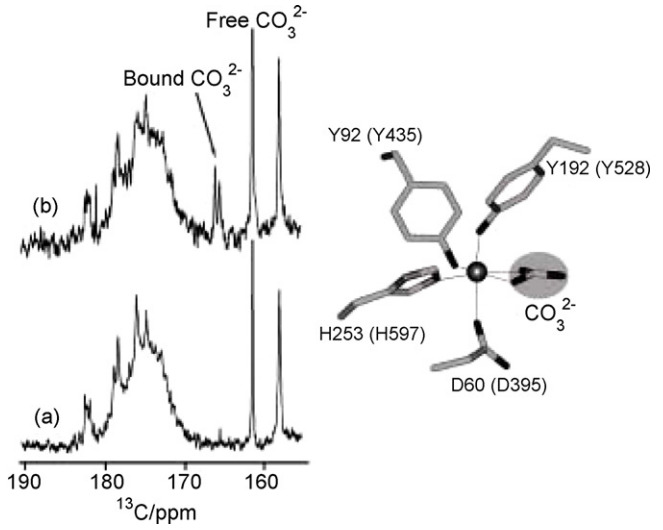


Fig. 7. ^{13}C NMR spectra of hLF (0.9 mM) in the presence of 10 mM $H^{13}CO_3^-$ (a), and with 2 mol equiv of [Bi(NTA)] (b), and the Fe(III)-binding site in the N-lobe (C-lobe) of human lactoferrin (*right*, PDBID: 1bol) (adapted with permission from Ref. [114]).

5.2.2. Human serum albumin

Bismuth compounds are almost non-toxic, and in general markedly less toxic than the analogues of arsenic and antimony which are in the same group with bismuth in the periodic table. The majority of cases of bismuth poisoning have occurred during medical therapy rather than industrial exposure. The potential toxicity of bismuth is normally diagnosed by the measurement of bismuth levels in blood or in blood plasma [117–119]. As the most abundant protein in serum (0.63 mM), human serum albumin (HSA) has been hypothesized to be the target of bismuth in plasma. The interaction of bismuth with human serum albumin was examined and the binding constant (log K_a) was measured to be 9–12 by fluorescent titration (Fig. 9) [104]. Chromatography (FPLC) in combination with ICP-MS data showed that 70.3% of bismuth associates to apo-hTF with the rest (29.7% bismuth) to HSA at HSA:apo-hTF:Bi (as ranrtidine bismuth citrate) = 26:1:2 and 13:1:2. However, almost all of the bismuth associates with HSA when the transferrin was saturated by Fe(III) (Fe_2-hTF), indicating that transferrin is a major target of bismuth in blood plasma [104].

5.2.3. Cysteine- and histidine-rich proteins

Metallothionein (MT) is a small protein with ca. 30% cysteine residues out of its ca. 60 amino acid residues (Fig. 10a) [120,121]. The cysteine-rich character of MT provides a high capacity of coordinating metal ions, such as Zn(II), Cu(I) and Cd(II). In addition to the function of metal storage (normally Zn(II) and Cu(I)) in biological systems, MT is able to donate Zn(II) to apo-enzymes, to control cell differentiation and proliferation, and to detoxify heavy metal ions such as Cd(II), Hg(II), and Au(I) [121,122]. Bi(III) is known to have a high affinity towards thiolate-containing molecules, and it binds to MT strongly with a stoichiometry of Bi:MT = 7:1 (Bi_7-MT). Bismuth coordinates to cysteines with Bi-S distances of 2.55 Å and additional oxygen coordination (Bi-O of 2.2 Å) was also

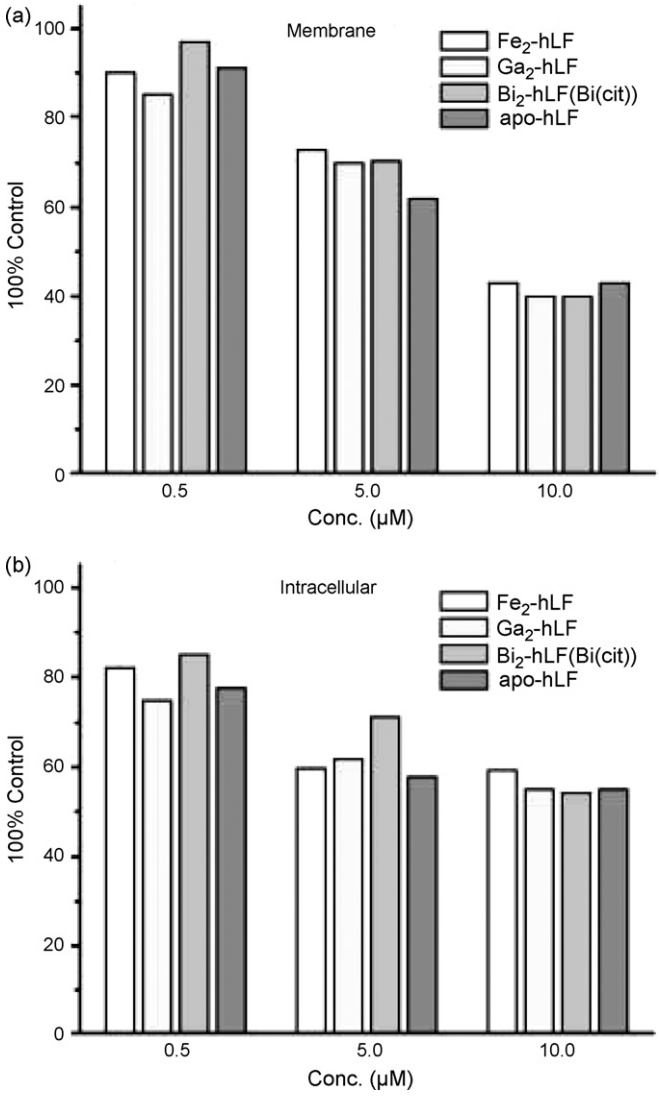


Fig. 8. Effect of increasing concentrations of metal-loaded lactoferrin on the uptake of ^{59}Fe-hLF complex by rat intestinal IEC-6 cells. Membrane-bound ^{59}Fe (a) and intracellular ^{59}Fe (b) (adapted with permission from Ref. [114]).

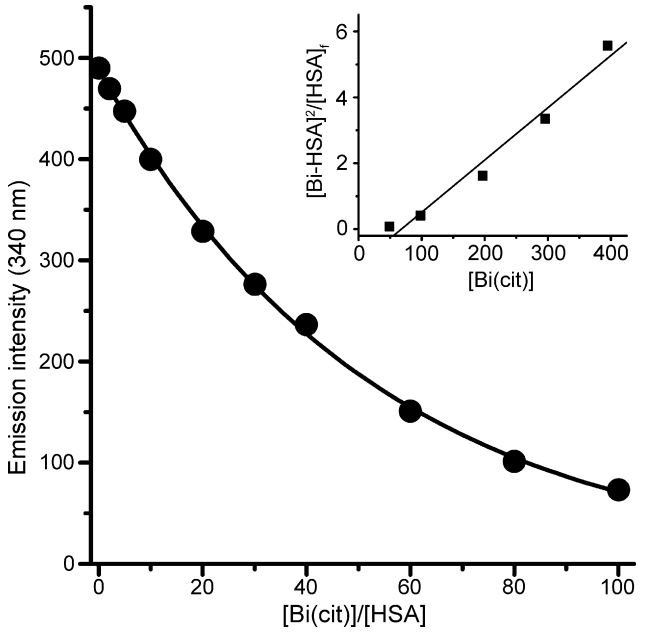


Fig. 9. Titration curve for Bi(III) binding to HSA. The decrease in fluorescence emission intensity at 340 nm is plotted vs. the [Bi(III)]/[HSA] ratio. Insert: plot of $[\text{Bi-HSA}]^2/[\text{HSA}]_f$ vs. [Bi(cit)] (adapted with permission from Ref. [104]).

observed. It can readily replace both Zn(II) and Cd(II) from the protein by a biphasic process [123]. ^{1}H NMR studies demonstrate that Zn(II) is replaced faster than Cd(II), and that both Zn(II) and Cd(II) are replaced much faster in the β-domain than that in the α-domain. Upon exposure of 50 μM bismuth citrate to macrophage cells at 12 and 24 h, metallothionein genes were found to be increased by 18- and 10-fold, respectively, representing the largest increase in gene expression [77]. The significant increase in metallothionein gene expression probably reflects a part of common defense and/or repair mechanisms following different stress stimuli.

Both UV–vis spectroscopy and ratiometric pulsed alkylation mass spectrometry (rPA-MS) show that bismuth coordinates to four cysteine residues in the metalloregulatory protein *S. aureus* plasmid pI258 CadC presumely tetrahedrally [124,125]. Each of the two Bi(III) sites in the homodimer of the protein consists of Cys7 and Cys11 near the N-terminus of one subunit and Cys58 and Cys60 in the putative α3-helix of the other. A *de novo* approach was used to investigate the interaction of heavy metals including Bi(III) with TRI family of peptides [126]. Binding of Bi(III) to these peptides and CadC were compared. The bond lengths of Bi-S in these complexes were determined to be 2.54 Å by EXAFS, similar to those in Bi_7-MT.

Both Hpn and Hpn-like (Hpnl) proteins from *H. pylori* are histidine-rich proteins with 28 and 19 histidine residues in Hpn (46.7%) and Hpn-like protein (25%), respectively (Fig. 10b) [127]. Moreover, both proteins present as multimers in solution and they may play a crucial role on intracellular nickel storage

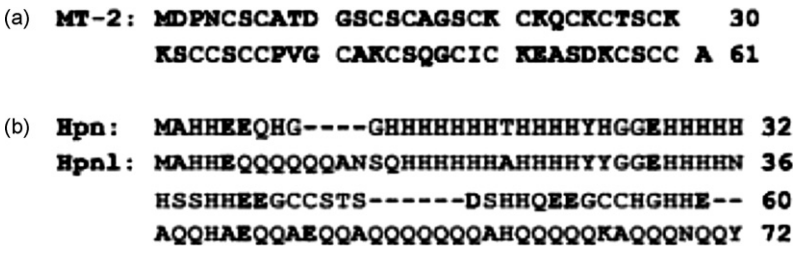


Fig. 10. The protein sequence of metallothionein (MT-2) (PDB ID: 4mt2) (a) and Hpn and Hpn-like protein (*H. pylori* 26695) [120,128] (b).

and homeostasis [128]. Mutagenesis experiments have shown that *H. pylori* with *hpn* gene knock-out are four-fold susceptible to bismuth antiulcer drugs than that of the wild-type [129], indicating a role of Hpn in *H. pylori* responses to the bismuth therapy, a common treatment for *H. pylori* infection. Hpn was overexpressed and purified recently, and the apo-protein binds to 3.81 (±0.23) Bi(III) ions per monomer with binding constant (K_d) of 11 μM [130]. At Bi(III) concentration above 30 μM, *E. coli* BL21 cells with the *hpn* gene on a pET plasmid were found to grow slightly better upon addition of IPTG than those without, whereas *E. coli* cells without the *hpn* gene grew much slowly than those with *hpn*. Strains with and without *hpn*, similarly exhibited different growth rates in the presence of Ni(II) (as $NiSO_4$). This suggests that Hpn plays a role in protecting *H. pylori* by sequestering excess intracellular bismuth ions [130]. In view of its short length (ca. 60 aa) and extremely high contents of potential metal-binding residues, Hpn may share some similarities with the zinc-storage protein, metallothionein [121]. Since many histidine-rich motifs and proteins are found in microorganisms, targeting such proteins may offer a novel approach for development of new antimicrobial and antiviral agents.

5.3. Interaction of bismuth with enzymes

5.3.1. Cytosolic alcohol dehydrogenase

Enzyme inhibition has long been thought to play a role in the mechanism of action of bismuth drugs [89,90,131]. The inhibition of cytosolic alcohol dehydrogenase (ADH) by bismuth drugs has been shown to suppress the production of acetaldehyde, which is toxic to mucosal cells [132], although the inhibition mechanism still remains unknown. Recently, inhibition and interaction of ADH by bismuth drugs were examined by using the baker's yeast alcohol dehydrogenase (YADH) as a model due to its high sequence identity to *H. pylori* ADH [133]. Bi(III) binds to cysteine residues on the enzyme as demonstrated by the increase in UV absorbance at 350 nm upon addition of Bi(III) (as Bi(cit)) (Fig. 11a). The interaction between Bi(III) and the enzyme exhibits a biphasic process and only one of the two Zn(II) ions can be substituted by Bi(III) (i.e., about one Zn(II) per monomer) (Fig. 11b). Since Bi(III) inhibits the enzyme activity in a non-competitive mode, the Zn(II) in the structural site may be replaced. Interestingly, the binding of Bi(III) to the enzyme gradually induces the enzyme dissociation, from its native form, a tetramer to a dimer. Inhibition of protein–protein interaction may represent one type of the modes of action of the metallodrug.

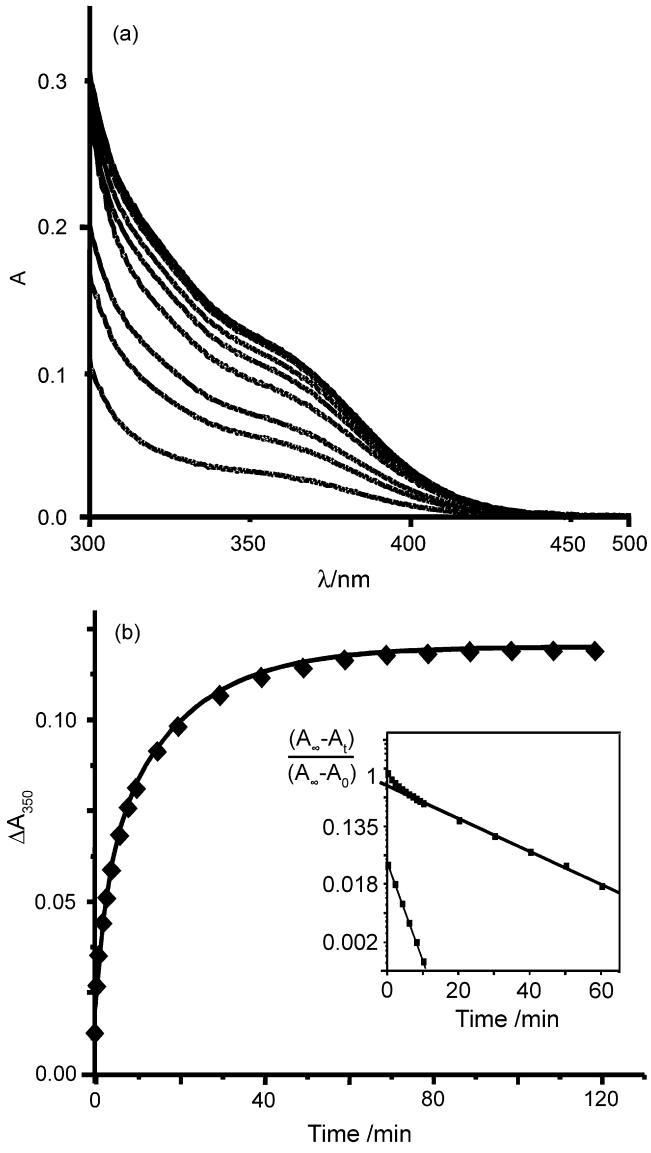


Fig. 11. UV absorbance of YADH in the presence of Bi(Cit) (a) and kinetics of the reaction of Bi(III) to YADH (b). Inset: semilog plot of the data and secondary plot of the fast step with a rate constant of 0.33 min^{-1} and slow step with a rate constant of 5.5×10^{-2} min^{-1} (adapted with permission from Ref. [133]).

5.3.2. Urease

Urease, an enzyme that converts urea into ammonia and carbonic acid, is crucial for *H. pylori* survival in the acidic environment of stomach [134]. Several reports have revealed that bismuth compounds can inhibit urease activity [135–137]. Bismuth complexes inhibit the activity of urease through either non-competitive (ranitidine bismuth citrate (RBC) or competitive (Bi(EDTA) and $Bi(Cys)_3$) mechanisms (Fig. 12a) [137]. Bismuth inhibits urease activity by blocking the enzyme active site *via* coordinating to the Cys319, a residue at the entrance of the enzyme active site. This was confirmed by comparing the wild-type urease with the C319A mutant *K. aerogenes* urease (Fig. 12b). The ability of bismuth drugs to inhibit urease depends on the stability of bismuth drugs, the weaker the bismuth compounds, the stronger the Bi(III) binds to the cysteine residues of enzyme *via* a ligand exchange. A series of triarylbismuthanes and their dthalides analogues was synthesized and structurally characterized. Some of these complexes exhibited inhibitory activities against jack bean urease. It seems that the inhibitory effects of these organobismuth complexes are not always governed by the Lewis acidity at the bismuth center [31]. The two most active complexes against urease, show no antifungal activity against *S. cerevisiae*, the same eukaryote as human, offering potentially new lead compounds for discovery of new bismuth antiulcer agents [31].

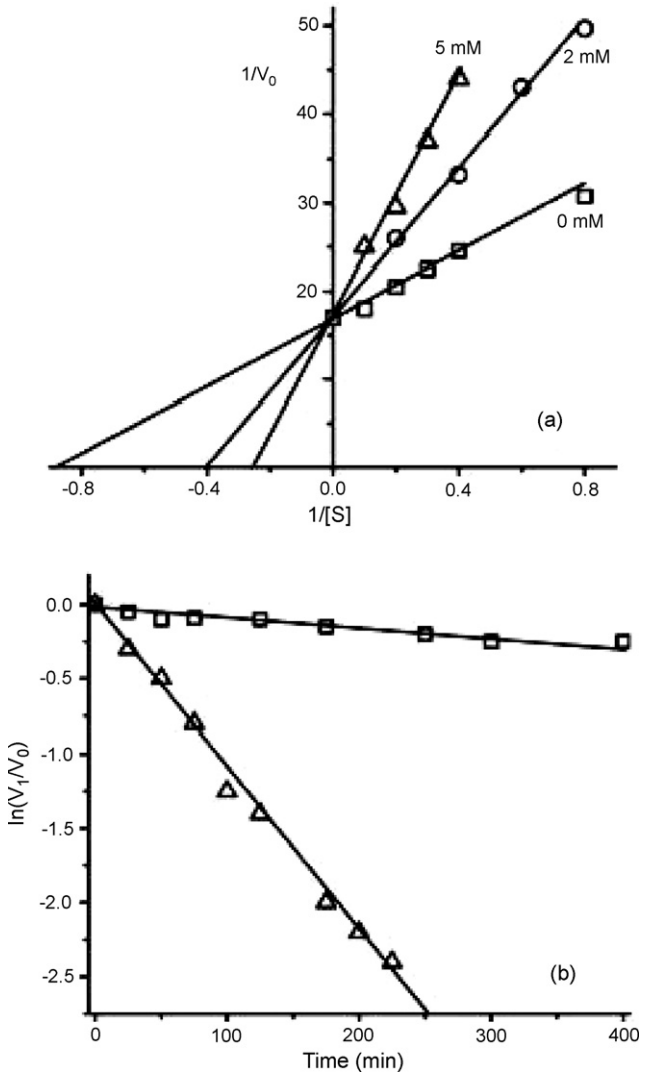


Fig. 12. Inhibition of jack bean urease by RBC (a) and kinetics of urease inactivation upon addition of Bi(EDTA) (b) Symbol code in (b): wide-type urease, (△); C319A mutant urease, (□) (adapted with permission from Ref. [137]).

6. Conclusions

Extensive chemical, biochemical and pharmacological studies of bismuth compounds have enabled the medical applications of clinically used bismuth compounds to be extended. Bismuth compounds offer potentials in cancer therapy not only by directly inhibiting the tumor growth (^{213}Bi) but also by indirectly reducing the side-effect of the clinically used anticancer drugs, i.e., cisplatin. It would be of interest for future work to identify binding or targeting proteins/enzymes of bismuth in biological systems (e.g., microorganisms) by metalloproteomics [138]. Further studies of recognition of Bi(III) with such biomolecules, will improve our understanding of the mechanism of action of bismuth drugs which may facilitate new metallodrugs to be designed. Some of this work has recently been reviewed elsewhere [139].

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