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14. ABSTRACT Different forms of heavy metals affect biochemical systems in characteristic ways that cannot be detected with typical metal analysis methods like atomic absorption spectrometry. Further, using living systems to analyze interaction of heavy metals with biochemical systems can be laborious and unreliable. To generate a reliable easy-to-use biologically-based biosensor system, the entire human metallothionein-II (MT-II) gene was incorporated into a plasmid (pUC57-MT) easily replicated in Escherichia coli. In this system, a commercial polyclonal antibody raised against human metal-responsive transcription factor-1 protein (MTF-1 protein) could modify the electrophoretic migration patterns (i.e. cause specific decreases in agarose gel electrophoretic mobility) of the plasmid in the presence or absence of heavy metals other than zinc (Zn). In the study here, heavy metals, MTF-1 protein, and polyclonal anti-MTF-1 antibody were used to assess pUC57-MT plasmid antibody-assisted electrophoretic mobility. Anti-MTF-1 antibody bound both MTF-1 protein and pUC57-MT plasmid in a non-competitive fashion such that it could be used to differentiate specific heavy metal binding. The results showed that antibody-inhibited plasmid migration was heavy metal level-dependent. Zinc caused a unique mobility shift pattern opposite to that of other metals tested, i.e. Zn blocked the antibody ability to inhibit plasmid migration, despite a greatly increased affinity for DNA by the antibody when Zn was present. The Zn effect was reversed/modified by adding MTF-1 protein. Additionally, antibody inhibition of plasmid mobility was resistant to heat pre-treatment and trypsinization, indicating absence of residual DNA extraction-resistant bacterial DNA binding proteins. DNA binding by anti-DNA antibodies may be commonly enhanced by xenobiotic heavy metals and elevated levels of Zn, thus making them potentially effective tools for assessment of heavy metal bioavailability in aqueous solutions and fluid obtained from metal implant sites.					
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RESEARCH ARTICLE

A plasmid containing the human metallothionein II gene can function as an antibody-assisted electrophoretic biosensor for heavy metals

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Abstract

Different forms of heavy metals affect biochemical systems in characteristic ways that cannot be detected with typical metal analysis methods like atomic absorption spectrometry. Further, using living systems to analyze interaction of heavy metals with biochemical systems can be laborious and unreliable. To generate a reliable easy-to-use biologically-based biosensor system, the entire human metallothionein-II (*MT-II*) gene was incorporated into a plasmid (pUC57-MT) easily replicated in *Escherichia coli*. In this system, a commercial polyclonal antibody raised against human metal-responsive transcription factor-1 protein (MTF-1 protein) could modify the electrophoretic migration patterns (i.e. cause specific decreases in agarose gel electrophoretic mobility) of the plasmid in the presence or absence of heavy metals other than zinc (Zn). In the study here, heavy metals, MTF-1 protein, and polyclonal anti-MTF-1 antibody were used to assess pUC57-MT plasmid antibody-assisted electrophoretic mobility. Anti-MTF-1 antibody bound both MTF-1 protein and pUC57-MT plasmid in a non-competitive fashion such that it could be used to differentiate specific heavy metal binding. The results showed that antibody-inhibited plasmid migration was heavy metal level-dependent. Zinc caused a unique mobility shift pattern opposite to that of other metals tested, i.e. Zn blocked the antibody ability to inhibit plasmid migration, despite a greatly increased affinity for DNA by the antibody when Zn was present. The Zn effect was reversed/modified by adding MTF-1 protein. Additionally, antibody inhibition of plasmid mobility was resistant to heat pre-treatment and trypsinization, indicating absence of residual DNA extraction-resistant bacterial DNA binding proteins. DNA binding by anti-DNA antibodies may be commonly enhanced by xenobiotic heavy metals and elevated levels of Zn, thus making them potentially effective tools for assessment of heavy metal bioavailability in aqueous solutions and fluid obtained from metal implant sites.

Keywords

Anti-DNA, anti-MTF-1, metal, metallothionein, MRE, MTF-1, plasmid, xenobiotic

History

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Introduction

Heavy metals are prototypical xenobiotics (Bigazzi, 1999; Gardner et al., 2010; Maecker et al., 2012; Pollard et al., 1999; Rowley & Monestier, 2005). A metal is characterized as a heavy metal if it is chemically classified as one of the 38 transition metals listed in the Periodic Table of the Elements. These types of metals include cobalt, chromium, zinc, nickel, and tungsten among others. Heavy metals are used in a variety of industrial processes and products, ranging from aircraft corrosion resistance to home plumbing. Heavy metals adversely impact living systems by interfering with normal physiologic functions, such as blood production, DNA repair, kidney function, calcium modulation, zinc finger enzyme activities, and immune system response to antigens. The usual route of exposure to heavy metals is via the respiratory system; exposure may occur during welding of metal

structural components, volatilization during anti-corrosion treatment of aircrafts and other vehicles, or during normal operation of metal-containing devices that may chronically release small amounts of metal substances into the ambient air. Heavy metal exposure may also occur by direct contact with the skin, causing a delayed-type hypersensitivity reaction (Kosboth et al., 2007). Heavy metals may also enter the food chain via contaminated water consumed by humans, animals, or plants, as well as via contaminated soil in which crop plants are grown. Among other effects, heavy metals induce the activity and production of cellular protective mechanisms or factors, which function to mitigate the toxicity of the offending heavy metal. Among the primary proteins in the cellular anti-heavy metal response are the metal-responsive transcription factor-1 (MTF-1) protein and metallothionein (MT) proteins.

The MTF-1 protein is the key component in the primary control mechanism for MT protein synthesis in mammalian cells (Heuchel et al., 1994; Seguin and Prevost, 1988; Westin & Schaffner, 1988). Organisms ranging from prokaryotes to humans utilize a super family of nearly universally occurring metal binding/chelating proteins, known as MT proteins, to protect cells from the deleterious effects of heavy metal exposure (Heuchel et al., 1994). MT proteins are low molecular weight (6–10 kDa), non-DNA binding proteins that specifically bind heavy metals in the cellular cytoplasm, thereby reducing their toxic effects (Haq et al., 2003; Klaassen et al., 1999; Miller et al., 2004; Palmiter, 1998).

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In contrast to MT proteins, MTF-1 protein is both a metal-binding and DNA-binding transcription factor that initiates MT protein synthesis by binding metal response elements (MREs) within promoters of metallothionein genes.

The gene for the human MTF-1 protein is located on the short arm of chromosome 1. The human MTF-1 protein sequence is strongly conserved across species, with 93% amino acid sequence identity to mouse MTF-1 protein (Brugnera et al., 1994; Heuchel et al., 1994; Lichtlen & Schaffner, 2001). The human *MTF-1* gene codes for an 81-kDa 753-amino acid protein normally localized in the cytoplasm but capable of translocating to the nucleus when an appropriate stimulus is provided. The current model of MTF-1 protein function suggests that the MTF-1 protein is induced to bind MREs when MT proteins in the cell cytoplasm bind heavy metals, which cause the MT proteins to release their stores of zinc ions (Waldron et al., 2009). The increased amounts of liberated zinc ions can then bind cytoplasmic MTF-1 protein by filling zinc fingers in the DNA binding regions of the cytoplasmic MTF-1 protein (Bittel et al., 1998; Dalton et al., 1997; Koizumi et al., 2000). On binding the zinc ions, the MTF-1 protein is then translocated to the nucleus where it binds specific DNA sequences in the metallothionein gene to induce synthesis of more MT protein that is then available to combat any increase in cytoplasmic heavy metal concentration (Sims et al., 2012).

The purpose of this study was to develop an entirely biologically based, low-cost bio-sensor system that could identify heavy metals in aqueous solutions and address the issue of heavy metal bioavailability in biological systems. For this purpose, the ability of several heavy metals to induce metal-specific DNA migration patterns in an agarose gel matrix was examined. This study examined the effects of cobalt, chromium, zinc, nickel, and tungsten. We report here the unique ability of zinc ions to greatly enhance anti-DNA antibody interaction with DNA, while simultaneously blocking the ability of a DNA-binding antibody to inhibit electrophoretic migration of a plasmid containing the entire human *MT-II* gene (pUC57-MT plasmid). This study also demonstrated the ability of MTF-1 protein to influence and further differentiate metal-specific agarose gel electrophoretic migration patterns of the pUC57-MT plasmid. Ultimately, this agarose gel-based method might be useful in heavy metal bioavailability testing of aqueous samples from a variety of sources (water treatment plants and natural and artificial bodies of water) and for heavy metal debris shed from medical and dental metal implants.

Materials and methods

All metals, buffers (biotechnology grade Tris, HEPES), phenylmethanesulfonyl fluoride (PMSF), molecular-grade glycerol, and BioUltra ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma (St. Louis, MO). All metals were 98–99.95% pure. The source DNA used as standard DNA for all experiments was a pUC57 plasmid from GenScript Corporation (Piscataway, NJ). The plasmid was custom-made to contain the full-length 837-bp human *MT-II* gene cloned into a pUC57 plasmid at the *EcoRV* restriction site within the multiple cloning site of the plasmid; thus, the *MT-II* gene was located between *BamHI* and *EcoRI* restriction endonuclease sites (this resulted in a 3547-bp plasmid). This ‘new’ plasmid was re-named pUC57-MT to further clarify that the plasmid contained the entire *MT-II* gene. Additional copies of the plasmid were grown in and extracted from an *Escherichia coli* vector (NEB 5-alpha Competent *E. coli* [High Efficiency], New England BioLabs, Ipswich, MA). Both versions of the plasmid were identical based upon endonuclease digest analysis and antibody-assisted electrophoretic mobility assays. When the plasmid was grown in-house, DNA extraction and

purification was performed using Wizard Plus SV Minipreps DNA Purification Systems (Promega, Madison, WI).

Human MTF-1 protein was obtained from Origene Technologies, Inc. (Rockville, MD). The MTF-1 had a predicted molecular weight of 80.8 kDa; purity was >80% (determined by gel electrophoresis and Coomassie blue staining). The MTF-1 protein was prepared by the vendor in 50- μ l aliquots at 0.27–0.51 mg/ml in storage buffer (25 mM Tris, 100 mM glycine, 10% glycerol [pH 7.5]), and stored at -80°C until use. All samples that contained MTF-1 protein were incubated on ice with 0.934 mg (934 ng) MTF-1 protein for 30 min, unless otherwise noted.

Anti-MTF-1 polyclonal antibody [anti-MTF-1(C-19)] was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibody is affinity purified goat polyclonal antibody raised against a peptide that maps near the carboxy-terminus of human MTF-1. The vendor supplied the antibody in 1-ml aliquots at 200 μ g IgG/ml phosphate buffered saline (with <0.1% sodium azide and 0.1% gelatin). All samples that contained antibody preparation were incubated on ice with 1 μ g (1000 ng) of anti-MTF-1 antibody for 20 min, unless otherwise noted. The basic composition of the reaction buffer used for all experiments consisted of a 2 $\times$ solution of Tris-HCl (200 mM Tris-HCl [pH 8.3]) diluted to 1 $\times$ on addition of other components (e.g. metal salts, protein, antibody, and/or plasmid).

Agarose gel electrophoresis used pre-cast E-Gels and EX-Gels with the E-Gel iBase system for real-time trans-illumination and the E-Gel Mini Power Base (Invitrogen, Carlsbad, CA). Ethidium bromide-containing gels were photographed using a Gel Logic 1500 Imaging System (Kodak, Rochester, NY). All electrophoresis was performed at room temperature (23°C), unless noted otherwise. DNA molecular size markers for agarose gel electrophoresis consisted of the E-Gel 1-kB Plus DNA Ladder obtained from Invitrogen. The ladder contained 20 DNA bands ranging in size from 1000–12,000 bp. Antibody pull-down assays were performed with a Melon Gel IgG Spin Purification Kit (Pierce Biotechnology, Rockford, IL).

Results

To determine metal specificity of the electrophoretic mobility of the pUC57-MT plasmid containing the human *MT-II* gene, agarose gel electrophoresis was performed at room temperature utilizing 0.8% agarose minigels. Figure 1 shows a control gel where none of the samples were treated with the anti-MTF-1 polyclonal antibody. In the absence of the antibody, no inhibition of mobility was observed, even when MTF-1 protein or MTF-1 protein and a heavy metal were present (Lanes 4–6). It is also seen that the heavy metals alone did not change the mobility of the pUC57-MT plasmid (Lanes 9 and 10). Addition of EDTA to the MTF-1 protein/heavy metal treated plasmid samples also had no effect on mobility in the absence of the anti-MTF-1 antibody (compare Lanes 5 and 6 to Lanes 7 and 8).

Figure 2(a) shows the results after incubating the plasmid with and without anti-MTF-1 antibody. This combination was further tested in the presence and absence of 2 mM ZnSO_4 or 2 mM NiCl_2 for 20 min on ice, followed by 30 min incubation on ice with or without MTF-1 protein. The control plasmid is shown in Lane 2 where the electrophoretic mobility of the 3547-bp plasmid was between those of the 3000- and 4000-bp DNA sizing bands shown in Lane 1. Lanes 3 and 4 show the mobility characteristics of the plasmid when incubated with the anti-MTF-1 antibody.

There was significant inhibition of mobility of the pUC57-MT plasmid in the presence of the anti-MTF-1 antibody. Lanes 5–7 of Figure 2(a) show the same mixture as in Lanes 3 and 4, but with addition of 2 mM ZnSO_4 . Addition of ZnSO_4 completely blocked

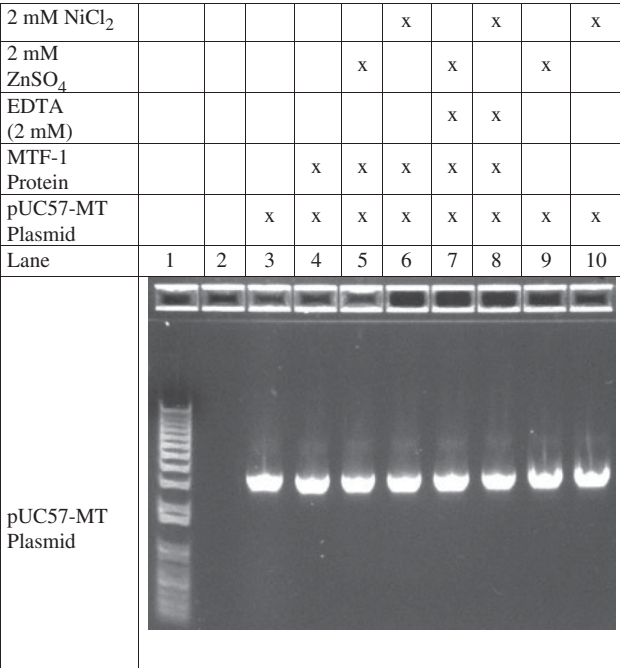


Figure 1. Control gel: antibody-assisted electrophoretic shift assay. Antibody-assisted electrophoretic shift assay using 0.8% agarose gel to determine migration patterns of the pUC57-MT plasmid (containing entire human *MT-II* gene) in the presence of zinc and nickel, with and without the MTF-1 protein and without anti-MTF-1 polyclonal antibody. Effects of heavy metals alone are shown in Lanes 9 and 10. No inhibition of plasmid electrophoretic mobility was seen under any conditions without the anti-MTF-1 antibody. Lane 1: 1kB Plus DNA sizing ladder.

the ability of the anti-MTF-1 antibody to inhibit plasmid mobility. However, addition of MTF-1 protein to the mixture reversed the effect of zinc and generated a different and distinct antibody inhibited pUC57-MT mobility pattern (Lanes 8–10). There was also a difference in plasmid mobility when the metal was changed from zinc to nickel (Lanes 11 and 12 versus Lanes 5–7). Unlike zinc, nickel did not block the ability of the antibody to inhibit mobility of the plasmid, but the mobility inhibition pattern was significantly different than that generated by the presence of the antibody alone (Lanes 3 and 4 versus Lanes 11 and 12). Figure 2(b) shows the results of tests with CoCl₂, which were compared to ZnSO₄ and NiCl₂ treatments. Treatment of the pUC57-MT plasmid with CoCl₂ generated an antibody-inhibited electrophoretic mobility pattern that resembled that of NiCl₂, but with greater magnitude.

Since there was a remote possibility that an endogenous bacterial DNA-binding protein could be resistant to the DNA extraction/purification process that the pUC57-MT plasmid underwent, samples of the isolated plasmid were pre-treated with heat (boiling for 45 s) and trypsinization to remove potential residual proteins. Figure 3 shows the electrophoretic mobility of the pUC57-MT plasmid in a 0.8% agarose gel after boiling in reaction buffer for 45 s. Boiling did not prevent the anti-MTF-1 antibody inhibition of mobility of the pUC57-MT plasmid under any subsequent conditions. Lane 1 of Figure 3 shows mobility of the plasmid without boiling and without a presence of anti-MTF-1 antibody. Lane 2 demonstrates that boiling did not change plasmid mobility. Lane 3 illustrates the typical response of non-boiled plasmid incubated with anti-MTF-1 antibody. Lane 4 of Figure 3 shows the typical change in mobility of the antibody-treated non-boiled plasmid when 2 mM ZnSO₄ was present. Lane 5 of Figure 3 shows the effect of NiCl₂ on the antibody-inhibited

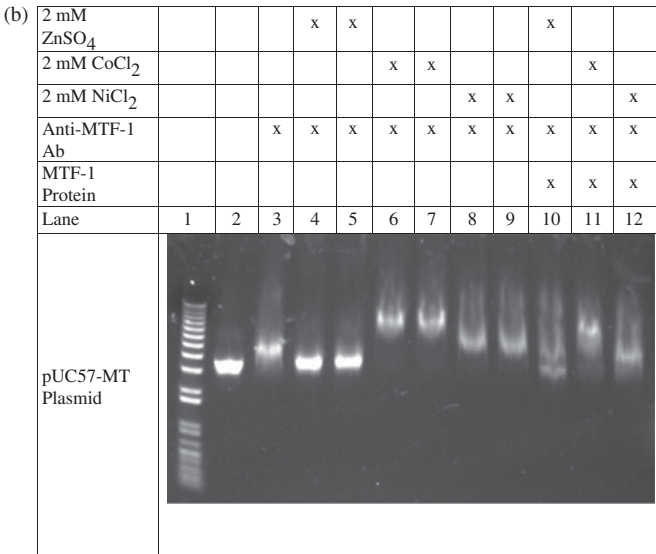
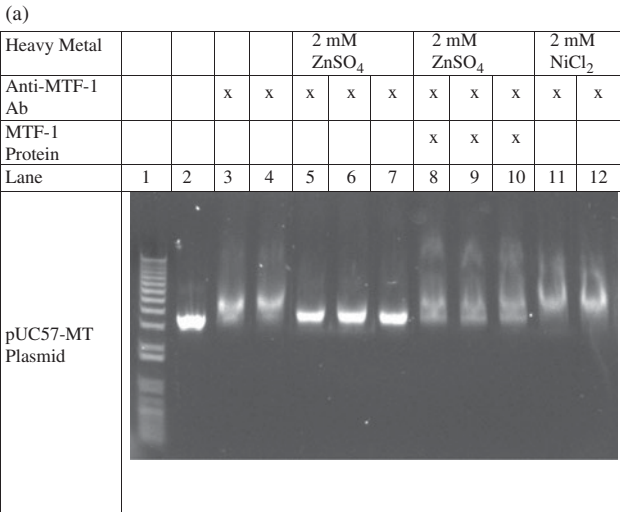


Figure 2. (a) Antibody-assisted electrophoretic shift assay using 0.8% agarose gel electrophoresis to determine migration patterns of the pUC57-MT plasmid (containing the entire human *MT-II* gene) in the presence of zinc and nickel, with and without the MTF-1 protein and anti-MTF-1 antibody. Agarose gel electrophoresis was performed using pre-cast 0.8% agarose gels containing ethidium bromide (EtBr). Lane 1: 1-kb Plus DNA sizing ladder. Lane 2: negative control (no antibody, no protein, no metal) containing only pUC57-MT plasmid. Zinc blocked the antibody ability to inhibit plasmid mobility; these effects were reversed by addition of MTF-1 protein (Lanes 3 and 4 versus Lanes 5–7 and Lanes 8–10). Nickel did not block antibody inhibition of plasmid mobility (Lanes 11 and 12) but, instead, enhanced the inhibition of mobility. The amounts of MTF-1 protein (when utilized), anti-MTF-1 antibody, and pUC57-MT plasmid used in each incubation tube were, respectively, 0.934 µg, 1.0 µg, and 0.288 µg. Final concentrations for MTF-1, antibody, and plasmid were 37.36, 40.00, and 11.52 ng/µl, respectively; these levels were used in all other experiments, unless otherwise noted. (b) Expanded antibody-assisted electrophoretic shift assay using 0.8% agarose gel electrophoresis to determine the migration pattern of the pUC57-MT plasmid (containing *MT-II* gene) in the presence of specific metals (zinc, cobalt, nickel), with and without MTF-1 and anti-MTF-1 antibody. Lane 1: 1-kb Plus DNA sizing ladder. Lane 2: negative control (no metal, no antibody) containing only plasmid. ZnSO₄ block-age of anti-MTF-1 antibody inhibition of plasmid electrophoretic mobility (Lanes 4 and 5) was reversed by addition of MTF-1 protein (Lane 10). CoCl₂ enhanced the ability of the anti-MTF-1 antibody to inhibit plasmid mobility more than NiCl₂ (Lanes 6 and 7 versus Lanes 8 and 9). MTF-1 protein caused small but distinct changes in mobility patterns of the pUC57-MT plasmid when exposed to cobalt and nickel (Lanes 11 and 12).

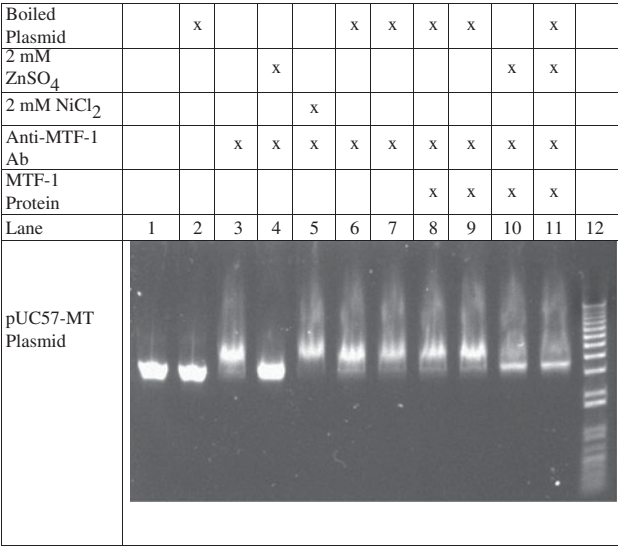


Figure 3. Plasmid boiling. Antibody-assisted electrophoretic mobility shift assay to test effects of boiling pUC57-MT plasmid for 45 s to potentially remove any residual protein attached to plasmid after DNA extraction and purification steps. Electrophoresis performed using pre-cast 0.8% agarose gels containing EtBr. Lane 1: negative control (no antibody, no protein, no metal, no boiling) containing only non-boiled plasmid. Pre-boiling the plasmid for 45 s did not prevent antibody-inhibition of electrophoretic mobility (Lane 2 versus Lanes 6–9 and Lane 11). MTF-1 protein was able to significantly reverse the effects of Zn even after a 45-s pre-boiling of the plasmid (Lane 4 versus Lanes 10 and 11). Lane 12: 1-kB Plus DNA sizing ladder.

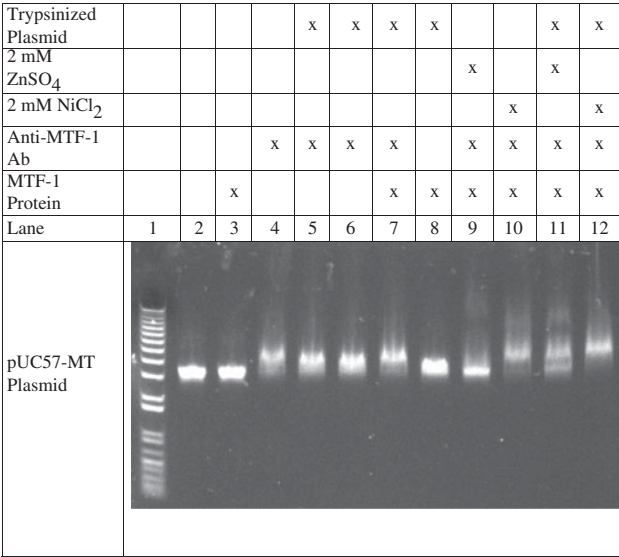


Figure 4. Plasmid trypsinization. Antibody-assisted electrophoretic mobility shift assay to test the effects of trypsin treatment of pUC57-MT plasmid for 1 h to potentially remove any residual bacterial DNA binding protein. Lane 1: 1-kB Plus DNA sizing ladder. Lane 2: negative control (no antibody, no protein, no metal, no trypsinization) containing only non-trypsinized plasmid. Lane 3: MTF-1 protein alone did not inhibit plasmid electrophoretic mobility. Trypsinization did not prevent antibody-inhibition of plasmid mobility; there was enhancement of electrophoretic mobility inhibition when MTF-1 was added (Lanes 5 and 6 versus Lane 7; Lane 9 versus Lane 11; Lane 10 versus Lane 12).

mobility of the non-boiled pUC57-MT plasmid. Lanes 6 and 7 of Figure 3 illustrate that the boiled plasmid retained the typical mobility inhibition in the presence of anti-MTF-1 antibody. Lanes 8 and 9 of Figure 3 demonstrate that antibody-inhibited mobility of the boiled pUC57-MT plasmid was not affected by addition of MTF-1 protein in the absence of ZnSO₄. Additionally, boiling the pUC57-MT plasmid did not eliminate the MTF-1 protein ability to reverse ZnSO₄ blockage of the antibody-inhibited mobility of the pUC57-MT plasmid (in Figure 3, compare Lanes 10 and 11 to Lane 4).

Further evaluation of the pUC57-MT plasmid after a 10-min trypsinization (0.02% trypsin, 0.04 mM EDTA) at 23 °C (prior to use) is shown in Figure 4. Trypsin activity was stopped by addition of PMSF (to final concentration of 1.8 mM). Lane 2 shows the pUC57-MT plasmid control. Lane 3 illustrates that MTF-1 alone had no effect on pUC57-MT electrophoretic mobility in the 0.8% agarose gel matrix. Comparing Lane 3 (non-trypsinized plasmid and MTF-1 protein) to Lane 8 (trypsinized plasmid and MTF-1 protein) reveals no significant difference between trypsinized and non-trypsinized plasmid mobility in the presence of the MTF-1 protein without the anti-MTF-1 antibody. Lane 4 illustrates the typical response of the plasmid to anti-MTF-1 antibody in the absence of zinc or any other heavy metal. Trypsinization of the plasmid did not prevent antibody inhibition of mobility (Lanes 5 and 6). As shown previously with the non-trypsinized plasmid, the MTF-1 protein had no effect on mobility of the trypsinized plasmid in the absence of anti-MTF-1 antibody (Lanes 7 and 8). Without antibody, no plasmid mobility changes were seen. Lanes 9 and 10 are positive controls for ZnSO₄ and NiCl₂ treatments in the presence of MTF-1 protein without trypsinization. Lanes 11 and 12 illustrate ZnSO₄ versus NiCl₂ mobility patterns with trypsinized pUC57-MT plasmid in the presence of MTF-1 protein and anti-MTF-1 antibody.

As heat treatment and trypsinization did not prevent antibody-inhibition of the mobility of the pUC57-MT plasmid, a direct antibody pull-down assay of the plasmid was performed in the presence and absence of zinc to further characterize antibody–plasmid interaction. Prior to any incubation, the plasmid was either cut into two fragments with *Bam*H1 and *Eco*R1 by digesting with the endonucleases for 1 h at 37 °C or left uncut (intact). The smaller fragment (865 bp) contained the entire *MT-II* gene (837 bp), including the MRE sequences, which are binding sites for the MTF-1 protein. The larger (2682 bp) fragment contained the remainder of the plasmid nucleotide sequence and no MRE sequences. The antibody pull-down assay (Figure 5) was performed utilizing a Melon Gel IgG Spin Purification Kit.

Uncut plasmid experiments are shown in Lanes 3, 4, and 5; *Bam*H1/*Eco*R1-digested pUC57-MT plasmid (cut pUC57-MT plasmid) experiments are shown in Lanes 7–12. The cut or uncut pUC57-MT plasmid was pre-incubated with or without the MTF-1 protein for 30 min in the presence or absence of ZnSO₄ at 4 °C. After pre-incubation of the plasmid with or without the MTF-1 protein, anti-MTF-1 antibody was added and the mixture was incubated for 20 min at 4 °C. The MTF-1 protein was always added 30 min before the antibody to ensure full binding of the MTF-1 protein to specific MRE binding sites in the *MT-II* gene within the pUC57-MT plasmid. The resulting antibody–DNA complexes were isolated by precipitation with cold acetone (1 h, –20 °C) and the precipitate was then re-suspended in 1 × reaction buffer without ZnSO₄. The ZnSO₄ was omitted after acetone precipitation since whatever was in the precipitate was already influenced/pulled down under ZnSO₄ conditions present in previous steps.

Figure 5 shows the effect of ZnSO₄ on affinity of the anti-MTF-1 antibody for pUC57-MT plasmid DNA. Lane 3 shows the pull-down results in the absence of the 2 mM ZnSO₄. Lane 4 demonstrates the dramatic effect of ZnSO₄ on the anti-MTF-1 antibody ability to bind directly to the plasmid DNA. Lane 5

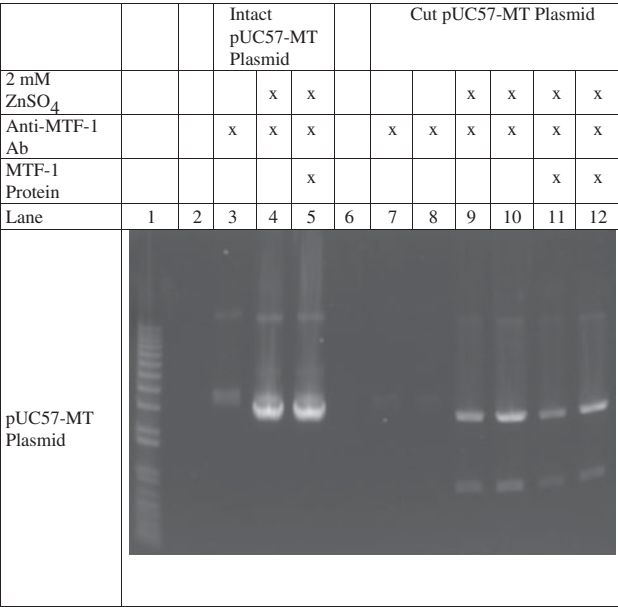


Figure 5. Antibody pull-down assay. Agarose (0.8%) gel electrophoresis showing anti-MTF-1 antibody pull-down assay of pUC57-MT plasmid in the presence of 2 mM ZnSO₄ before (lanes 3–5) and after endonuclease digestion (lanes 7–12). Each sample had an anti-MTF-1 antibody present for pull-down assay. Electrophoresis was performed using 0.8% agarose gels containing EtBr. Starting amounts of MTF-1 protein (when utilized), anti-MTF-1 antibody, and plasmid DNA in reaction solution were 1.87, 8.0, and 0.575 µg, respectively. MTF-1 was always added 30 min before adding the antibody to ensure full binding of MTF-1 to binding sites. Lane 1: 1-kB Plus DNA sizing ladder. Lanes 2 and 6: blank (no plasmid, antibody, metal, or protein).

shows that MTF-1 protein did not interfere with the ability of the anti-MTF-1 antibody to bind pUC57-MT plasmid DNA. There was no decrease in antibody-DNA binding in the presence of the MTF-1 protein. Lanes 3–5 illustrate that ZnSO₄ greatly increased the affinity of the antibody preparation for the uncut plasmid DNA.

When *Bam*-*H*I and *Eco**R*I cut the pUC57-MT plasmid into two unequal size fragments, the anti-MTF-1 antibody was still able to bind both fragments with much higher affinity in the presence of ZnSO₄ (Figure 5). Lanes 7 and 8 show the antibody pull-down results that occurred when ZnSO₄ was absent. Both fragments of the endonuclease-treated plasmid were bound with far less affinity (Lanes 7 and 8) when compared to the same assay performed with addition of ZnSO₄ (Lanes 9 and 10). Addition of MTF-1 protein did not significantly alter the ability of the antibody to bind the two fragments of endonuclease-treated pUC57-MT plasmid DNA (Lanes 11 and 12).

To further characterize the interaction between the anti-MTF-1 antibody and pUC57-MT plasmid, plasmid antibody-assisted electrophoretic mobility was examined in the presence and absence of the metal ion chelator EDTA (Figure 6). Lane 3 shows a typical electrophoretic migration of the plasmid alone in a 0.8% agarose gel. Lane 4 shows the migration pattern of the plasmid in the presence of MTF-1 protein. This again showed that MTF-1 protein alone was not able to significantly alter the electrophoretic mobility of the plasmid, even though the latter contained the entire *MT-II* gene, including specific binding sites (MRE) for the MTF-1 protein.

As also depicted in Figure 6, Lane 5 demonstrates the typical electrophoretic response of the pUC57-MT plasmid to the anti-MTF-1 antibody. Lane 6 shows the effect of adding MTF-1 protein to the plasmid preparation in the presence of 2 mM ZnSO₄. A comparison of Lane 6 to Lane 8 illustrates the effect of

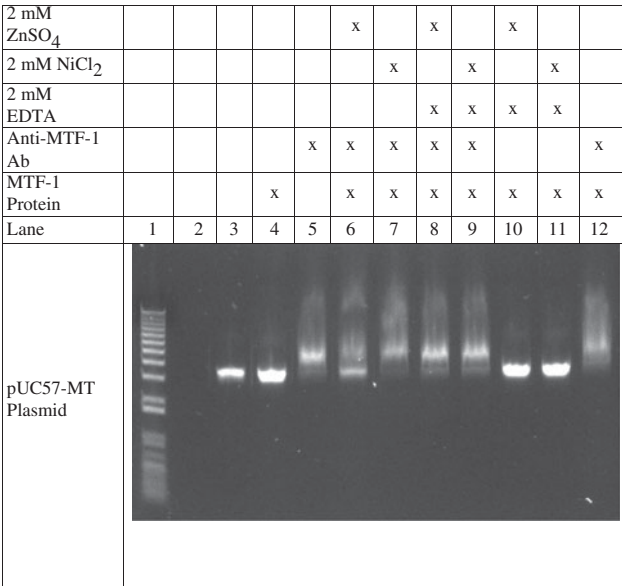


Figure 6. EDTA treatment. Antibody-assisted electrophoretic mobility shift assay in 0.8% agarose gels containing EtBr testing effects of zinc and nickel in the presence/absence of EDTA. Lane 1: 1-kB Plus DNA sizing ladder. Lane 2: blank (no plasmid, metals, protein, antibody, or EDTA). Lane 3: negative control (no metal or antibody) containing only pUC57-MT plasmid. Lane 4: pUC57-MT plasmid with MTF-1 protein only. Lane 5: pUC57-MT plasmid with anti-MTF-1 antibody only. Lanes 6–12: noted in associated table. EDTA eliminated differences between effects from ZnSO₄ and NiCl₂ treatment, but had no effect on plasmid mobility in the absence of anti-MTF-1 antibody.

EDTA on the ability of ZnSO₄ to alter the mobility of the plasmid. Lane 8 shows that 2 mM ZnSO₄ was far less able to block antibody inhibition of pUC57-MT plasmid mobility when 2 mM EDTA was present. The pattern in Lane 8 closely resembles that in Lane 5; the effect of ZnSO₄ was negated by the addition of EDTA. Lanes 7 and 9 show the effect of EDTA on the ability of NiCl₂ to alter mobility of the pUC57-MT plasmid. EDTA was also able to block the NiCl₂ effect on plasmid mobility in the presence of the antibody, converting it to that typically seen with the antibody alone (compare Lane 9 versus Lane 5). EDTA was able to partially restore plasmid mobility as seen by enrichment of the lower band in Lane 9 compared to the same band area in Lane 7. Lane 12 shows the combined effects of MTF-1 protein and anti-MTF-1 antibody; Lane 6 shows that ZnSO₄ increased plasmid mobility in the combined presence of MTF-protein and MTF-1 antibody. Lane 7 shows that NiCl₂ further inhibited plasmid mobility under the same conditions. However, the plasmid in Lane 12 did not show the same mobility pattern as the EDTA-treated samples in Lanes 7 and 8. Apparently, there is some residual heavy metal influence on plasmid mobility in the presence of EDTA. This further suggests high affinity interactions between the heavy metals and the anti-MTF-1 antibody and/or the MTF-1 protein.

To determine the concentration ranges of the heavy metal effects on the antibody-assisted electrophoretic mobility of the pUC57-MT plasmid, the plasmid was treated with progressively decreasing concentrations of metals (i.e. 2, 1, 0.5, and 0.25 mM). Figure 7 shows the plasmid response to progressively decreasing concentrations of ZnSO₄, NiCl₂, CrCl₃, and WCl₆. Metal concentration dependence is shown in each case. Consistent with previous experiments, ZnSO₄ was the only metal that had a concentration effect that was opposite that of all other metals tested, i.e. as ZnSO₄ levels decreased, the electrophoretic mobility of the pUC57-MT plasmid decreased in the presence

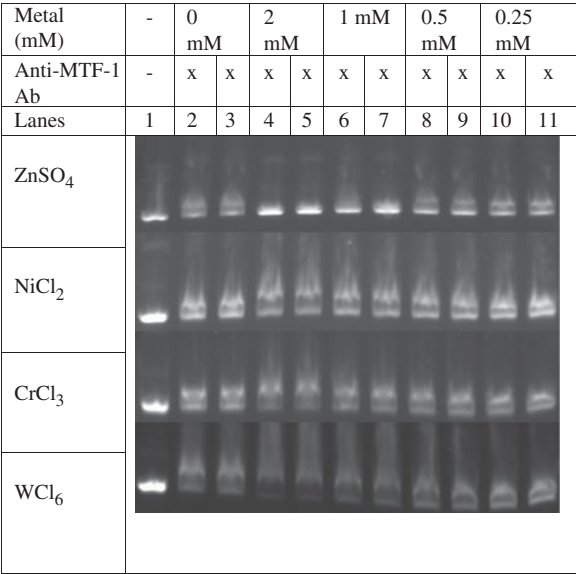


Figure 7. Heavy metal concentration dependence of the pUC57-MT antibody-assisted electrophoretic mobility shifts. Heavy metal concentration was varied from 0.25–2.00 mM in the presence of anti-MTF-1 antibody (Lanes 4–11). Each metal concentration is shown in duplicate. Lane 1: negative control (i.e. no metal, no antibody). Lanes 2 and 3: positive controls (all cases). Electrophoretic mobility of plasmid increased in the 0.8% agarose gel as respective levels of NiCl₂, CrCl₃, and WCl₆ decreased. However, as the ZnSO₄ concentration decreased, the mobility of the plasmid decreased. ZnSO₄ blockage of anti-MTF-1 antibody inhibition of plasmid electrophoretic mobility was decreased as the ZnSO₄ concentration decreased.

of the anti-MTF-1 antibody. The antibody was able to more easily inhibit mobility of the pUC57-MT plasmid as ZnSO₄ levels decreased. All other metals tested allowed increased mobility as their concentration decreased, i.e. as the concentration of NiCl₂, CrCl₃, and WCl₆ decreased, electrophoretic mobility of the plasmid increased despite a presence of anti-MTF-1 antibody. Lower concentrations of the Ni, Cr, and W ions were less able to enhance antibody inhibition of the pUC57-MT plasmid mobility. Cobalt results (data not shown) were similar to those of Cr, Ni, and W.

The promoter region of metallothionein genes contain MREs that function in induction of MT protein synthesis when the MREs are bound by MTF-1 protein. The MREs contain the core consensus DNA sequence TGCRCNC (Serfling et al., 1985; Stuart et al., 1985; Westin & Schaffner, 1988), where R represents a purine (adenine or guanine) nucleotide and N represents any nucleotide. The next set of experiments (as depicted in Figure 8) examined the effect of anti-MTF-1 antibody and MTF-1 protein on a 25-bp double-stranded (ds) oligonucleotide (MREaLy; sequence in figure) containing an MRE sequence that interacts with human MTF-1 protein. Like the endogenous MRE sequences found in *MT* gene promoters, MREaLy has the core TGCRCNC consensus sequence. The electrophoretic mobility of the oligonucleotide through a 2% agarose gel was significantly affected by a presence of specific metals. ZnSO₄ and CrCl₃ were tested at 100 μM; other metals (nickel, cobalt, tungsten) yielded similar results regardless of incubation at room temperature (23 °C) or 4 °C for 30 min prior to electrophoresis. Figure 8 shows the results from the incubations at 4 °C. Similar to the pUC57-MT plasmid, the anti-MTF-1 antibody was able to inhibit the mobility of the MREaLy. Unlike its effect on pUC57-MT plasmid, the MTF-1 protein was also capable of inhibiting mobility of the oligonucleotide, but to a lesser extent than the anti-MTF-1 antibody (compare Lane 2 versus Lane 3).

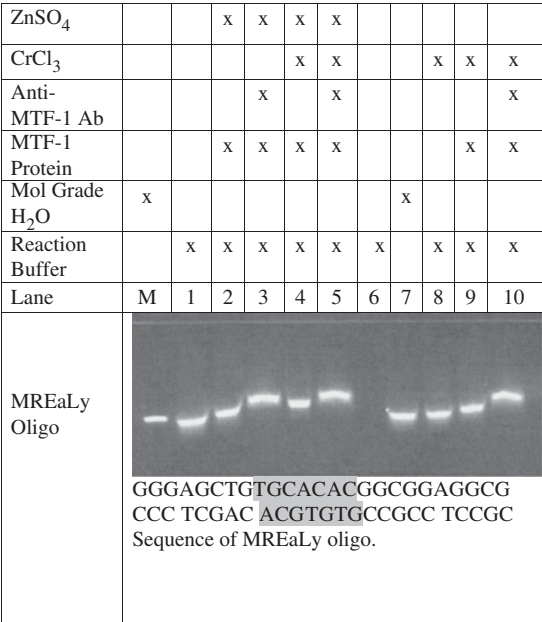


Figure 8. Zinc and chromium effects on electrophoretic mobility of MREaLy oligonucleotide. Antibody-assisted electrophoretic mobility assay (in 2% agarose gel containing EtBr) of oligonucleotide containing MRE sequence. Oligonucleotide was pre-incubated with 100 μM ZnSO₄, 100 μM CrCl₃, or both metals. Lane M was usually reserved for DNA sizing standards, but in this case was used as a sample lane instead. The region in the gel where 25-bp MREaLy migrated without any additions is labeled 'MREaLy Oligo'. Full DNA sequence of MREaLy used in electrophoretic mobility assays is also shown. The MRE core consensus sequence is shaded in gray; non-shaded sequences are more variable flanking regions.

The effect of ZnSO₄ and CrCl₃ on the mobility of MREaLy is shown in Figure 8, where ZnSO₄ had the same effect as CrCl₃ (compare Lane 3 versus Lane 10). ZnSO₄ and CrCl₃ effects were additive in the presence of MTF-1 protein and without anti-MTF-1 antibody (compare Lane 4 versus Lane 2 which shows the effect of only ZnSO₄). Overall, the effects of the MTF-1 protein and the anti-MTF-1 antibody were additive in the presence of ZnSO₄. This is unlike their effects on the pUC57-MT plasmid, where the MTF-1 protein reversed effects of ZnSO₄ in the presence of the antibody. While CrCl₃ alone did not inhibit MREaLy mobility (Lane 8), addition of MTF-1 protein with the CrCl₃ resulted in significant mobility inhibition (Lane 9). Lastly, as with the ZnSO₄, the effect of MTF-1 protein and anti-MTF-1 antibody were again cumulative in the presence of CrCl₃ (Lane 10).

Discussion

MTF-1 protein is the key component in the primary control mechanism for metallo-thionein (MT) protein synthesis in mammalian cells (Heuchel et al., 1994). The current model of the action of MTF-1 protein indicates that it serves as a sensor of intracellular metal ion levels by binding free zinc (Zn) ions in the cytoplasm (Bittel et al., 1998; Dalton et al., 1997). The Zn ion concentration increases when extracellular heavy metals enter the cell and displace Zn ions from their normal intracellular binding sites. Upon binding the increased Zn ions, the MTF-1 protein is translocated into the nucleus where it binds directly to MRE sequences in *MT* gene promoter regions. This ultimately activates synthesis of messenger RNA that is translated into MT protein in the cytoplasm, adding to the heavy metal defense of the cell.

Utilizing a plasmid that contains the entire human *MT-II* gene (pUC57-MT), we evaluated the ability of an antibody preparation to change the electrophoretic migration of the plasmid under

specific heavy metal and MTF-1 protein conditions. We sought to identify heavy metal-specific pUC57-MT plasmid electrophoretic migration patterns that could be utilized to identify specific heavy metals and assess the bioavailability of the heavy metals. We determined that MTF-1 protein alone was not able to cause a detectable change in electrophoretic mobility of the plasmid in the absence of a polyclonal anti-MTF-1 antibody. However, the MTF-1 protein alone was able to alter/inhibit the electrophoretic mobility of the 25-bp MREaLy oligonucleotide that contained an MRE sequence (Figure 8). In that case, effects of Zn and Cr ions were the same or additive (Figure 8). Additionally, although the mobility of the MREaLy was inhibited by both the MTF-1 protein and anti-MTF-1 antibody (individually and combined), it cannot be used to identify *specific* heavy metals. The oligonucleotide combined with MTF-1 and anti-MTF-1 antibody would seem to only be an effective indicator of a *presence* of biologically active heavy metal(s) and so could potentially be used as an alternate simpler biosensor to assess the general presence of a heavy metal form that is biologically active.

There was some concern about the remote possibility of an endogenous bacterial DNA-binding protein surviving the plasmid DNA extraction/purification procedure such that it was still bound to the plasmid, thereby cross-reacting with the anti-MTF-1 antibody and preventing binding. Tests were performed to determine if the anti-MTF-1 antibody was binding to a residual Zn-sensitive bacterial DNA binding protein or directly to the DNA. Bacteria are known to have Zn-sensitive transcription repressors that dissociate from their DNA binding sites or change their protein conformations when free Zn ion levels increase due to Zn displacement by toxic heavy metals (Morby et al., 1993; Morita et al., 2002; Wang et al., 2010). These transcription repressors are responsible for preventing continuous production of metal binding proteins in the absence of an increased metal threat in bacteria. Boiling and trypsinization of the pUC57-MT plasmid did not eliminate the ability of the anti-MTF-1 antibody to inhibit the electrophoretic mobility of the pUC57-MT plasmid (Figures 3 and 4). This indicated the plasmid was free of any residual endogenous bacterial DNA-binding proteins and that anti-MTF-1 antibody was binding directly to the pUC57-MT plasmid DNA.

When the pUC57-MT plasmid was cut into two restriction fragments with *Bam*H1 and *Eco*R1 endonucleases, the anti-MTF-1 antibody preparation was able to bind both fragments in a Melon Gel antibody pull-down assay. The hypothesis that Zn ions caused an anti-MTF-1 antibody cross-reacting Zn-sensitive bacterial repressor protein to dissociate from previously DNA-extracted pUC57-MT plasmid was not supported by the findings here. Contrary to the residual bacterial repressor protein hypothesis, Zn ions greatly increased the binding of anti-MTF-1 antibody to the plasmid and the Zn-enhanced antibody binding was not altered by the presence of MTF-1 protein. Zinc-enhanced binding by the antibody occurred with both the intact plasmid and the endonuclease-digested plasmid.

Additionally, a heavy metal-induced dimerization of the antibody preparation was not supported by the Melon Gel antibody pull-down data. The Melon Gel results showed that increased amounts of DNA were bound in the presence of Zn ions, even though these ions caused the pUC57-MT plasmid to migrate farther into the agarose gel. Simple antibody dimerization should impede DNA-antibody complex migration due to the increased mass when compared to the DNA alone or DNA plus antibody experiments. If the other heavy metals caused antibody dimerization, the effect would be irrelevant to the development of a DNA-protein heavy metal biosensor, as long as the electrophoretic migration patterns of the DNA-protein complex were specific for individual types of heavy metals, and also reproducible.

Addition of EDTA eliminated the effect of Zn ions and caused the electrophoretic mobility of the pUC57-MT plasmid to be either very similar to that of any of the other non-Zn ions that were tested or very similar to the anti-MTF-1 antibody-only results. The data here showed that Zn ions had a unique effect on electrophoretic mobility of the plasmid, i.e. its effect was opposite that of the other metals tested. Although all metal effects (including Zn ions) were concentration-dependent, Co, Cr, W, and Ni ions enhanced the anti-MTF-1 antibody inhibition of the plasmid mobility in manners relatively similar to each other (when compared to zinc), but individually distinguishable. The mobility patterns of the Zn and non-Zn metal ions had significant variations that may be useful in identification of specific heavy metals and in the assessment of heavy metal bioavailability via an agarose gel electrophoresis method that does not require expensive complex equipment, especially when tests are performed in the presence of the MTF-1 protein.

To further characterize the effects of Zn ions and other metal ions on electrophoretic mobility of the pUC57-MT plasmid, a double-stranded (ds) 25-bp oligonucleotide was prepared that contained the MRE core sequence of TGCACAC. Figure 8 showed that MTF-1 alone was capable of inhibiting electrophoretic mobility of the 25-bp MRE-containing oligonucleotide. However, the addition of anti-MTF-1 antibody greatly enhanced the inhibition of the mobility of the oligonucleotide. This further supported the suggestion that the anti-MTF-1 antibody and the MTF-1 protein did not competitively bind to the same site on the plasmid and, therefore, have a synergistic effect on the inhibition of the mobility of the plasmid.

With the exception of Zn ions, all other metal ions tested in the antibody-assisted electrophoretic mobility shift assay enhanced the ability of anti-MTF-1 antibody to inhibit the pUC57-MT plasmid mobility. The metal-enhanced mobility inhibitions suggested that the heavy metals increased antibody affinity for DNA binding sites and the metal-specific electrophoretic mobility patterns may be related to the number of individual antibodies bound to the dsDNA and conformational changes the DNA experiences on antibody binding. This enhanced mobility inhibition was seen with the intact pUC57-MT plasmid, the endonuclease-digested plasmid, and the 25-bp oligonucleotide containing the MRE sequence. However, further testing of Zn ions in the antibody pull-down assay showed that even Zn induced higher affinity DNA binding by the antibody, even though the Zn ions actually blocked the ability of anti-MTF-1 antibody to inhibit the mobility of the plasmid. All data reported here strongly suggest the anti-MTF-1 antibody preparation did not compete with MTF-1 protein during DNA binding. The sites for the two types of DNA binding proteins (antibody and MTF-1 protein) were apparently separate and even showed synergistic effects.

Conformational changes in the dsDNA may be a key factor in the ability of Zn ions to block inhibition of plasmid electrophoretic mobility by the anti-MTF-1 antibody. Since Zn clearly enhanced antibody binding to the plasmid DNA, instead of causing the antibody to dissociate from the DNA, the Zn-enhanced binding may actually have caused the plasmid to assume a more compact conformation of a covalently closed circular DNA, e.g. supercoil, spiral writhe structure, twist, etc. The more compact conformation may then have caused the Zn-DNA-antibody complex to migrate further into the agarose gel, despite a greater collective mass. The addition of MTF-1 protein may have induced a more open dsDNA conformation that restored but modified the antibody-inhibited mobility of the plasmid. This hypothesis will have to be tested further by more direct examination of DNA structural changes in the presence of Zn ions, antibody, and MTF-1 protein.

Since all antibody-assisted electrophoretic mobility shift assays were performed at room temperature (23°C), there is strong evidence the plasmid DNA-metal-antibody-MTF-1 protein complex was very stable at room temperature. Additionally, the oligonucleotide experiments done at 4°C and 23°C had very similar outcomes, even though the experiments (including preparation of proteins, DNA, antibody, and reaction buffer) were entirely maintained at either 4°C or 23°C from start to finish.

Conclusions

This report demonstrated a direct analysis of the functional interactions of several industrial and militarily relevant heavy metals with a plasmid containing the human *metallothionein-II* gene, its transcriptional co-activator (human MTF-1 protein), and a metal-sensitive DNA binding polyclonal antibody. Naturally occurring components were utilized to form a useful biologically-based heavy metal analysis method that addresses heavy metal bioavailability, i.e. whether a heavy metal is in a form that can interact with the actual components of the cellular heavy metal defense system. Complex analytical methods like atomic absorption spectrometry cannot directly distinguish between forms of heavy metals that are biologically active (bioavailable) and those that are biologically inert. This agarose gel method may fill the gap between detection and assessment of bioavailability. Additionally, as Zn ions and other heavy metal ions have specific effects on the binding affinity of DNA-binding antibodies, some DNA-binding antibodies can be specifically utilized to isolate DNA from a mixture of proteins and nucleic acids simply by varying the zinc ion concentration. Extensive biochemical characterization of the anti-MTF-1 antibody showed that the anti-MTF-1 antibody, or a subset of the polyclonal mixture comprising the antibody preparation, bound directly to the pUC57-MT plasmid instead of to a residual Zn-sensitive DNA-extraction-resistant endogenous bacterial protein. This finding adds to the utility of the antibody preparation and the MTF-1 protein in the testing of heavy metal bioavailability in a biologically relevant system.

Bioavailability testing may be particularly useful after atomic absorption spectrometry testing of water samples from the environment or elsewhere has revealed a presence of heavy metals. This agarose gel-based bioavailability assay could add valuable insight into the ability of the contaminating heavy metal to interact with key biological proteins that protect cells from heavy metal poisoning. The gel-based method may be utilized in water treatment facilities or on samples from bodies of water that have been identified as contaminated with heavy metals after testing with atomic absorption spectrometry or other biologically-destructive methods (e.g. isotope dilution mass spectrometry, inductively coupled plasma mass spectrometry).

Additionally, this agarose gel-based method may be useful in medical/dental implant testing to determine if forms of heavy metals that may be released from metal implants are capable of interacting with biological systems. Many metal implants corrode and degrade over time and shed metallic debris that may cause adverse effects. It may be useful to determine which metal implants produce the most biologically reactive corrosion/degradation products based on bioavailability of the forms of heavy metal debris generated. The agarose gel-based method provides a rapid and inexpensive method of accomplishing bioavailability testing and identifying specific heavy metals.

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Declaration of interest

The authors report no conflicts of interest. The authors have no financial or personal conflicts of interest that could inappropriately influence this work.

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