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ARTICLE

Assessing bioavailability and genotoxicity of heavy metals and metallic nanoparticles simultaneously using dual-sensing *Escherichia coli* whole-cell bioreporters

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Abstract Genetically engineered bacterial strains called whole-cell bioreporters (WCBs) generated by fusing the promoter region of stress-responsive genes and reporter genes have been widely used as biosensors to detect toxic materials in the environment. In this study, we report a dual-sensing WCB harboring *recAp*::*egfp* and *zntAp*::*mcherry* to measure the genotoxicity and bioavailability of heavy metals and metallic nanoparticles (NPs) simultaneously. Since the dual-sensing WCB harbored *recAp*::*egfp* and *zntAp*::*mcherry*, the genotoxicity and bioavailability of heavy metals and metallic NPs that activate ZntR would be assessed by measuring the fluorescence signal of enhanced green fluorescent protein (eGFP) and mCherry, respectively. Among the tested heavy metal(loid)s, only Cr induced both eGFP and mCherry expression, and some of them only induced mCherry, thereby suggesting that Cr is genotoxic. In case of the tested metallic NPs, Ti_2O NPs, ZnO NPs, and Au NPs showed weak inhibitory effects on growth, but the eGFP was not induced. It was inferred that the tested NPs were not genotoxic and the inhibitory effects would not be related to direct DNA damage pathways. In addition, it was observed that ZnO NPs induced mCherry expression, indicating that the Zn ion was dissolved from the NPs. Although the dual-sensing WCB described here was limited to ZnO NPs, WCBs would be an alternative tool to investigate the dissolution of metallic NPs when the corresponding metal ion sensing systems were available.

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Keywords Bioavailability · Chromium · Genotoxicity · Whole-cell bioreporter · ZnO nanoparticles

Introduction

The inflow of many industrial pollutants into the environment is a major public health concern. Since the accumulation of pollutants causes adverse effects on living organisms, including humans, it is important to understand the mechanisms of the toxic effects. Generally, the toxicity of pollutants, including metals and metallic nanoparticles (NPs), is assessed using diverse model systems such as earthworms, plants, animals, and human cell lines (Fabrega et al. 2011; Sytar et al. 2013; Heggelund et al. 2014; Kwak et al. 2014; Lee and An 2015; Lee et al. 2015). However, it is not clear how the metals and NPs induce toxic effects in model systems because each organism utilizes unique mechanisms to respond to different stresses.

The stresses caused by heavy metal(loid)s, metallic NPs, and diverse chemicals on living organisms can be assessed by determining their total amount in the media by instrumental analysis (Ramaswamy et al. 2011; Storelli and Barone 2013). However, instrumental analysis cannot provide information on bioavailability because the total amount of pollutants is not biologically active for living organisms. In other words, a risk assessment based on total amount does not take the different behaviors of pollutants in each environment into consideration. To overcome this shortcoming, genetically engineered bacterial cell-based biosensors, which can measure the fraction of pollutants that actually affects bacterial cells, were developed and have been used widely (Baumann and van der Meer 2007; Hynninen and Virta 2010; van der Meer and Belkin 2010; Song et al. 2014). In most cases, the bioavailable fraction is less than the total

amount of pollutants because the tight association with the environmental media converts pollutants into non-bioactive states (Ivask et al. 2004; Maderova and Paton 2013; Yoon et al. 2016). The mechanism of the bacterial cell-based biosensor, called whole-cell bioreporters (WCBs), involves the fusion of the promoter regions of stress-responsive genes and reporter genes. When the WCBs are exposed to specific pollutants, the transcription of the reporter genes under the promoters is initiated. Moreover, the expression of reporter genes is proportional to the concentration of the bioavailable portion of pollutants; thus, WCBs are widely used as biosensors to quantify bioavailable fractions. For example, the reporter genes under *zntAp*, a promoter region of zinc-responsive genes in *E. coli*, are induced by diverse metal ions such as Cd(II), Zn(II), Pb(II), and Hg(II) because the conformational change of ZntR, a regulatory protein, upon metal binding allows the transcription of *zntA* (Changela et al. 2003; Gireesh-Babu and Chaudhari 2012).

The adverse effects of pollutants on living organisms occur via diverse cellular pathways resulting in cytosolic toxicity and genotoxicity. A toxic effect of a pollutant initiated by DNA damage is defined as genotoxicity, and the pollutant is called a genotoxin. Generally, genotoxic effects are reversed in a living organism by SOS repair systems that are initiated by the expression of *recA* genes (Kenyon et al. 1982; Sassanfar and Roberts 1990). To the same extent, the genotoxicity of pollutants has been assessed using WCBs produced by fusing the promoter regions of genotoxic-responsive genes and reporter genes such as *umuC-lacZ*, *sulA-lacZ,* and *recA-lux* (Oda et al. 1985; Min et al. 1999; Norman et al. 2005). Moreover, oxidative stress has also been assessed using WCBs produced by fusing genes involved in the oxidative repair system and reporter genes such as *katG-lux* (Mitchell and Gu 2004).

The objective of this study was to elucidate the mechanisms of the adverse effects of heavy metalsand metallic NPs on *Escherichia coli* using a dual-sensing WCB harboring *recAp::egfp* and *zntAp::mcherry*. Since the transcription of *egfp* and *mcherry* in the dual-sensing WCB was controlled by *recAp* and *zntAp*, genotoxicity and bioavailability could be assessed by measuring the expression of eGFP and mCherry, respectively. In the present study, we investigated the genotoxic effects and bioavailability of heavy metal(loid)s including Zn, Cd, As, Cr, Pb, Ag, and Ni and metallic NPs, including ZnO, Au, and TiO_2 NPs.

Materials and methods

Bacterial strains and chemicals

E. coli DH5α was used as a host strain for gene cloning and for WCBs. The WCBs were grown in lysogeny broth (LB). The tested heavy metal(loid)s ($CdCl_2$, $K_2Cr_2O_7$, $HgCl_2$, $NiCl_2$, $AsCl_3$, $PbCl_2$, AgCl, and $ZnCl_2$) and metallic NPs, ZnO NPs (particle size < 50 nm), TiO_2 NPs (particle size 21 nm), and Au NPs (particle size 20 nm), were purchased from Sigma-Aldrich (St. Louis, MO, USA). The properties of these NPs were characterized and reported previously (Nam et al. 2013). Mitomycin C (MMC) was used as a reference for genotoxin and purchased from Abcam (Cambridge, MA, USA).

Plasmid construction

The promoter regions of *rec*A (*recAp*) and *znt*A (*zntAp*) employed as sensing domains were amplified by PCR using *taq* polymerase (iStarTaq, iNtRON, Seungnam-si, Korea) from the genomic DNA of *E. coli* DH5α prepared by an alkaline lysis method supplemented with lysozyme (Sambrook et al. 1989). The reporter genes *egfp* and *mcherry* were from pEGFP-N1 and pmCherry-1 plasmids (Clontech, Seoul, Korea), respectively. The sequences of the primers used for PCR are listed in Table 1. The fusion of *recAp–egfp* was performed by two-step PCR (Good and

Table 1 Sequences of primers used for plasmid construction

Genes	Direction	Restriction enzyme site	Sequence (5′–3′)
recAp	Forward	*Bgl*II	GT**AGATCT**GCCACTGCCCGCGGTGAAGG
	Reversed	–	ATGTATAT***CTCCTTC***TTATACTTAACTAATATACTAA GATGCCGGGTAATACCGG
eGFP	Forward	–	CTTAGTATATTAGTTAAGTATAA***GAAGGAG***ATATAC ATATGGTGAGCAAGGGCGAGG
	Reversed	*Xho*I	AT**CTCGAG**TTACTTGTACAGCTCGTCCATGC
zntAp	Forward	*Bgl*II	AC**AGATCT**CGGCCTGCTACTTTGCC
	Reversed	*Xba*I	CC**TCTAGA**GGGCTTTCTTGCCGTGAT
mCherry	Forward	*BamH*I	GT**GGATCC**ATGGTGAGCAAGGGCGAG
	Reversed	*Xho*I	AT**CTCGAG**CTTGTACAGCTCGTCCATGC

Bold letters indicate the restriction enzyme sites

The underlined italic letters indicate the ribosome binding sites

Nazar 1992; Xiong et al. 2004). Briefly, the PCR fragments of *recAp* and *egfp* were obtained in the first PCR step, and the second PCR step was performed using the mixture of *recAp* and e*gfp* fragments as templates with the forward primer of *recAp* and the reverse primer of *egfp*. The PCR product of *recAp–egfp* was inserted into pCDFDuet-1 (Novagen, Madison, USA) using the *Bgl*II and *Xho*I restriction enzyme sites. The *E. coli* ribosome binding site sequence (GAAGGA) was added between the promoter and *egfp*, and it is indicated in bold italic letters in Table 1. The plasmid for sensing heavy metal bioavailability was obtained by inserting *zntAp* using the *Bgl*II and *Xba*I sites to replace the T7 promoter in the pET21(a) vector (Novagen), and *mcherry* was then inserted downstream of *zntAp* using *Bam*HI and *Xho*I. The sequences of the two plasmids, named pCDFDuet–recAp–eGFP and pET–zntAp–mCherry, were confirmed by DNA sequencing, and both plasmids were then transformed into *E. coli* DH5α to generate WCBs cable of sensing both bioavailability and genotoxicity.

Measuring the toxicity by WCB assay

The WCBs harboring both plasmids, pET–zntAp–mCherry and pCDFDuet–recAp–eGFP, were grown overnight at 37 °C in LB containing 100 and 50 μg/mL ampicillin and spectinomycin, respectively, and the overnight culture was given fresh LB medium. The WCB cells were exposed to different concentrations of heavy metals (Cd, As, Cr, Pb, Zn, Ag, and Ni) and metal NPs (ZnO NPs, TiO_2 NPs, and Au NPs) when the optical density of cells at 600 nm (OD_{600}) reached about 0.4. The overall toxicity was assessed by measuring OD_{600}, and the portion of bioavailable heavy metal ions, such as Zn, Cd, Pb, and Cr, was determined by measuring the intensity of mCherry. The genotoxicity was assessed by measuring the intensity of eGFP. MMC, which is a well-known genotoxic chemical that causes DNA damage, was used as a reference for the genotoxicity assay. For the measurement, 0.5 mL of the WCBs exposed to toxic materials was collected at different exposure durations, and the LB medium was removed by centrifugation. The cell pellets were resuspended in 1 mL of 50 mM Tris–HCl (pH 7.6) containing 160 mM KCl before the measurement. The OD_{600} was measured with a UV/Vis spectrophotometer, and the intensities of eGFP and mCherry were measured with a fluorescence spectrophotometer (FC-2, Scinco, Seoul, Korea) equipped with a xenon lamp as a light source and bandwidth-adjustable filters for excitation and emission. The bandwidth was fixed at ±5 nm, and the wavelengths for excitation/emission were 470/510 nm and 570/610 nm for eGFP and mCherry, respectively.

Data analysis

All experiments were performed more than three times, and the data are presented as the means and standard deviations as errors. The fluorescent signals of eGFP and mCherry from WCBs are presented as specific fluorescence (SF) defined as [the intensity of fluorescent proteins in WCBs induced by exposure/the optical density of WCBs (OD_{600})] to normalize the growth inhibition of *E. coli* by exposure to the toxic materials (Van Dyk et al. 1994; Min et al. 1999). For the bioavailability of heavy metals, the values are presented as an induction coefficient defined as [the mCherry intensity of exposed WCBs/the mCherry intensity of unexposed WCBs] (Yoon et al. 2016).

Results and discussion

Growth inhibition and genotoxic effects

To investigate the toxic and genotoxic effects of heavy metal(loid)s, the optical density at 600 nm and the expression level of eGFP at 480/510 nm of excitation/emission wavelengths were measured by UV/Vis spectrophotometer and fluorescence spectrophotometer, respectively. From the concentration-dependent tests, it was noticed that the growth of *E. coli* was inhibited completely at 1 mM and higher concentration of metal(loid)s and the expression of eGFP was not observed (data not shown). Because of this, 0.5 mM was selected as the concentration of metal(loid)s to compare the genotoxicity between diverse metal(loid)s. In addition, 1 μM of MMC was tested as a reference for the genotoxic material. As shown in Fig. 1, the OD_{600} and the eGFP expression levels of WCBs were measured at different time points up to 18 h after the addition of heavy metal(loid)s. Most of the metals including Cd, Ag, Zn, Pb, and Ni showed weak inhibitory effects on *E. coli* growth, while the MMC, As, and Cr showed significant inhibition over 50 % (Fig. 1A). Simultaneously, the expression level of eGFP indicating the genotoxic effects was also measured by FC-2 fluorescence spectrophotometer at 470/510 nm for the excitation/emission wavelengths (Fig. 1B). The intensity of eGFP generally increased with exposure duration, but most metal(loid)s showed weaker signals than the WCBs without metal exposure (control). However, MMC and Cr induced the expression of eGFP in the WCBs suggesting that Cr was a genotoxic material like MMC. Moreover, it was speculated that the adverse effects on *E. coli* of MMC and Cr would be caused via DNA damage. As shown in Fig. 1(A), the inhibitory effects of Cr and MMC on cell growth were significant. Therefore, it should take the

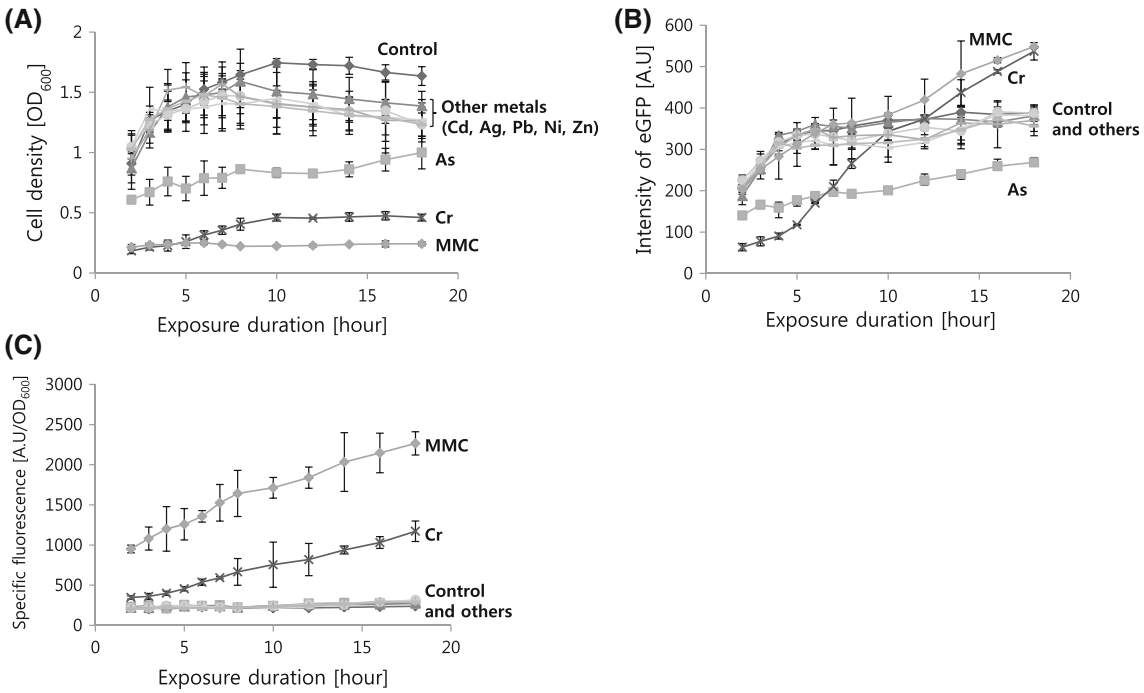

Fig. 1 Toxic effects of heavy metal(loid)s on *E. coli* WCBs. (A) Growth curves of WCBs exposed to heavy metal(loid)s. The growth curves indicating toxic effects on *E. coli* were obtained by measuring the OD_{600} over 18 h. (**B**) Fluorescence signal of eGFP that was induced by the exposure of WCBs to heavy metal(loid)s. The induction of eGFP indicating genotoxic effects was measured by fluorescence spectrometry using 480/510 nm excitation/emission wavelengths. (**C**) SF values of WCBs exposed to heavy metal(loid)s. The SF was defined as the fluorescence signal of eGFP divided by the OD_{600} of WCBs to normalize the inhibitory effects of heavy metal(loid)s on growth. MMC was used as a reference genotoxin

amount of WCB cells into account to assess the genotoxic effect represented as the expression level of eGFP. To rule out the different inhibition rates of diverse metal(loid)s, the SF similar to the 'specific bioluminescence (SBL)' mentioned in previous reports (Min et al. 1999) was introduced. The SF value was defined as [the intensity of eGFP (Arbitrary Units)/the optical density of WCBs (OD_{600})] and the corrected expression level of eGFP was shown in Fig. 1(C). It was noticed that MMC, a reference genotoxin, induced the expression of eGFP in WCB cells. This result was in concordance with the general notion that MMC is a genotoxic chemical and confirmed that the WCB-based *recAp–egfp* fusion was able to sense genotoxic effects. Moreover, only Cr from the tested metal(loid)s showed an increase in SF values in a time-dependent manner, indicating that Cr is a genotoxic chemical.

The genotoxicity of heavy metals has long been the subject of intense investigation because heavy metal contamination is a concern worldwide (Jose et al. 2011; Tchounwou et al. 2012; Thompson et al. 2012). Genotoxicity is generally studied by analyzing DNA damage in model systems. However, it was not effective for assessing the genotoxicity of heavy metals accumulated in diverse environmental media. On the other hand, the WCB assay would be an alternative to overcome the shortcomings in the current approaches, especially, in the aspects of time-saving, inexpensiveness, and convenience. Recently, the genotoxicity of the toxic materials including Cr in contaminated soils was assessed using an *Acinetobacter baylyi* strain harboring ADP1-recA-lux (Song et al. 2014). Although the experimental conditions were different, it supported the capability of WCBs described here to assess the genotoxicity of toxic materials.

Bioavailability of heavy metals

Since the WCB harbored not only *recAp::egfp* but also *zntAp::mcherry*, the genotoxicity and bioavailability of metals would be assessed simultaneously. In this case, the transcription of *mcherry* is controlled by ZntR, a regulatory protein bound to zntAp region, and ZntR allows the transcription in the presence of metal ions such as Zn, Cd, Pb, Co, Hg, and Ni (Beard et al. 1997; Gireesh-Babu and Chaudhari 2012). Moreover, it was observed that Cr also induced the expression of mCherry in this WCB system from the preliminary tests.

The results in the previous section showed that only Cr showed genotoxic effects among tested metal(loid)s, and Pb, Zn, and Cd showed weak inhibitory effects on growth rather than genotoxic effects. Thus, it was necessary to

determine whether the different inhibitory effects were caused by different cellular mechanisms or entrance of metallic ions into the cells. To verify this idea, the WCBs were exposed to 0.5 mM of Cd, Zn, Pb, and Cr and 1 μM of MMC, and then the intensity of mCherry was measured at different time points during 10 h (Fig. 2A). It was noticed that all the tested metals except MMC induced mCherry expression and the induction rates of mCherry were slightly different from that of each metal ion. It was reasoned that the strength of the interactions between the metal ions and ZntR caused these differences (Brocklehurst et al. 1999; Binet and Poole 2000), thereby inferring that the order of the ZntR association strength was Cd > Zn > Cr > Pb. In addition, the expression of eGFP was also measured and converted to SF values at the same time to elucidate the relationship between bioavailability and genotoxicity (Fig. 2B). Among the tested heavy metals, Cr induced the expression of both eGFP and mCherry, while the others induced only mCherry, as found in the previous section. MMC was used as a reference genotoxin and only induced eGFP. Consequently, it was concluded that the genotoxic effect of metallic ions was not due to the entrance into WCB cells but the genotoxic nature of Cr.

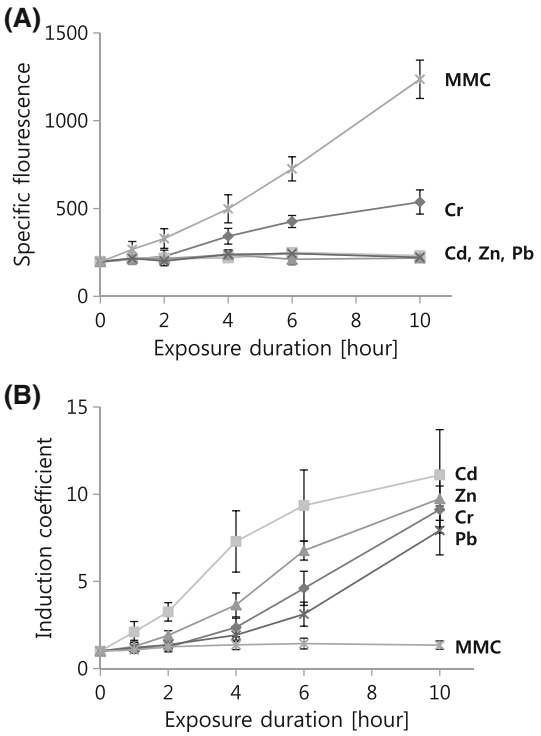

Fig. 2 Relationship between the bioavailability and genotoxicity of heavy metals. (**A**) The SF of eGFP from WCBs exposed to heavy metal ions and MMC was plotted against exposure duration. MMC was used as a reference genotoxin. (**B**) The induction coefficient of mCherry of WCBs exposed to heavy metals and MMC. The fluorescence mCherry signal was measured at 570/610 nm excitation/emission wavelengths

Genotoxicity of Cr on *E. coli*

To verify the concentration dependency of the Cr genotoxicity, the WCBs were exposed to 0–1 mM Cr and 1 μM MMC as a reference. The WCBs exposed to different concentrations of Cr were collected at different exposure times, and the OD_{600} and eGFP intensity were measured using a UV/Vis spectrophotometer and fluorescence spectrophotometer, respectively. As shown in Fig. 3(A), 0.01–0.1 mM Cr had weak inhibitory effects on the growth of WCBs, while 0.5 and 1.0 mM inhibited the growth significantly. Because of this growth inhibition, the nascent intensity of eGFP could not represent the genotoxic effect and it was not correlated with the concentration of Cr (Fig. 3B). To exclude the different amount of cells, the fluorescent intensity and optical density values were converted to SF described in the previous section (Fig. 3C). As a result, the genotoxic effect of Cr was observed clearly after 5-h exposure from 0.1 mM. When the SF values at 15-h exposure were compared, it was noticed that the genotoxic effects were increased with the higher concentration of Cr (Fig. 3D). In fact, the genotoxicity of chromium compounds has been reported previously by analyzing direct DNA damage (Bianchi and Levis 1984, 1988; De Flora et al. 1990). Nonetheless, assessing the genotoxicity of heavy metals using WCBs would be a useful tool. Moreover, our results confirmed the genotoxic nature of Cr reported in the previous studies, suggesting that the WCB assay would be an alternative tool to investigate the genotoxicity of pollutants.

Toxic effects of metallic NPs

Since the dual-sensing WCB system was able to measure the genotoxic effect of heavy metals, metallic NPs were also investigated. As shown in Fig. 4, the genotoxicity of metallic NPs, including ZnO NPs, TiO_2 NPs, and Au NPs, was investigated. The OD_{600} and intensity of eGFP of WCBs exposed to 1 mM ZnO, TiO_2, or/and Au NPs were measured at different exposure durations. After 24 h of exposure, the inhibition of cell growth was indicated by a decrease in the OD_{600} value of about 30 % by ZnO NPs, 15 % by Au NPs, and 21 % by TiO_2 NPs (Fig. 4A). However, a genotoxic effect (expressed as the SF of eGFP) was not observed except for MMC (Fig. 4B). This result agreed with previous reports on the genotoxicity of NPs (Trouiller et al. 2009; Yang et al. 2009; Nam et al. 2013). Although these studies employed different genotoxicity assays, genotoxicity was not observed for metallic NPs such as ZnO, Au, and Ag NPs in chromotests (Nam et al. 2013) but was observed for carbon nanotubes (Yang et al. 2009). It was reported that TiO_2 NPs showed adverse effects via a secondary genotoxic mechanism associated

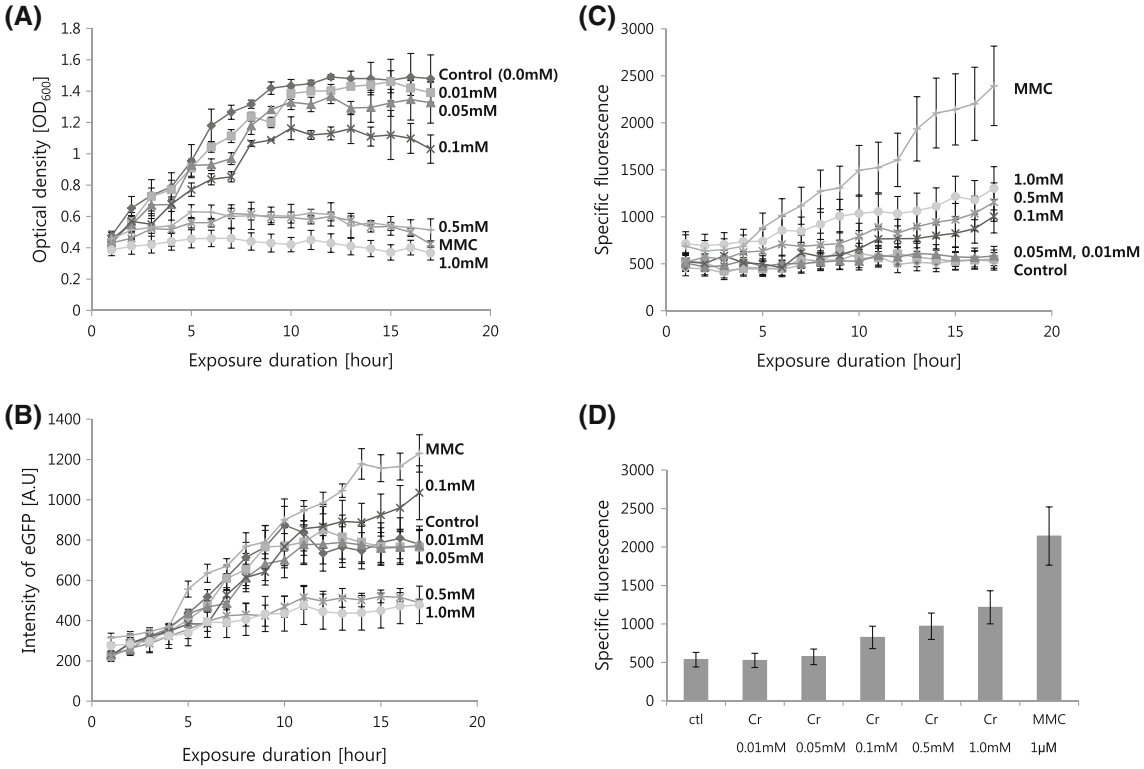

Fig. 3 Genotoxic effects of Cr assessed by WCBs. (**A**) Inhibitory effects of Cr on cell growth measured by OD_{600}. (**B**) Intensity of eGFP in WCBs exposed to different concentrations of Cr. (**C**) Normalized concentration-dependent genotoxic effects of Cr represented by SF. (**D**) Concentration-dependent SF values of WCBs at 15-h exposure. MMC was used as a reference genotoxin

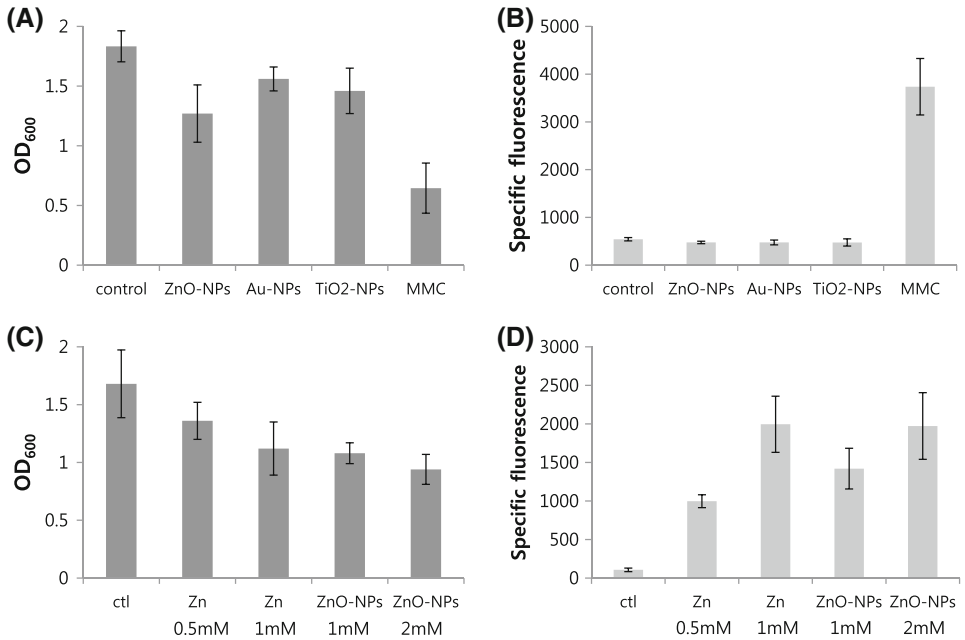

Fig. 4 Toxic effects of metallic nanoparticles, including ZnO NPs, on *E. coli*. The metallic NPs, including ZnO, Ti_2O, and Au NPs, were added to WCBs, and the OD_{600} and fluorescence intensity were measured after a 10-h exposure. (**A**) OD_{600} of WCBs exposed to 1 mM metallic NPs. (**B**) Expression of eGFP indicating genotoxic effects of metallic NPs. (**C**) Growth inhibition of WCBs by $ZnCl_2$ (0.5 and 1 mM) and ZnO NPs (1 and 2 mM). (**D**) SF of mCherry indicating the presence of bioavailable Zn ions from $ZnCl_2$ (0.5 and 1 mM) and from ZnO NPs (1 and 2 mM)

with inflammation and oxidative stress (Trouiller et al. 2009). Moreover, the toxicities of metallic NPs in living organisms differed according to their characteristics, such as size, shape, surface chemistry, and substance (Bian et al. 2011; Misra et al. 2012; Lee and An 2013; Nam et al. 2014). Therefore, it is important to take these factors regarding NPs into consideration when assessing the risks of metallic NPs.

Since the WCBs studied here can detect the presence of heavy metal ions such as Zn, Cr, Cd, and Pb, they were also applied to investigate the dissolution of ZnO NPs. The dissolution of ZnO NPs has been studied, and it was reported that the dissolved Zn ions were the main reason for the toxic effects on living organisms (Cho et al. 2011; Xia et al. 2011; Kim and An 2012). To examine the dissolution rate of ZnO NPs, the WCBs were exposed to Zn ions and ZnO NPs, and the optical density (OD_{600}) and the SF values of mCherry were measured (Figs. 4C, D). By comparing the SF values, the dissolved Zn ions from ZnO NPs could be measured qualitatively. As a result, the Zn ion level from 1 mM ZnO NPs was higher than that from 0.5 mM and less than that from 1 mM $ZnCl_2$, and the level for 2 mM ZnO NPs was similar to that for 1.0 mM $ZnCl_2$ (Fig. 4D). However, the overall toxicity of ZnO NPs as determined by cell growth was higher than that of $ZnCl_2$, as shown in Fig. 4C, thereby indicating that the nanotoxicity of NPs contributed to part of the toxicity. In summary, the results indicate that the toxicity of ZnO NPs was caused by both dissolved ions and NP toxicity.

In the present study, we investigated the genotoxicity and bioavailability of heavy metals and metallic NPs using dual stress-responsive WCBs harboring pCDFDuet–recAp–eGFP and pET–zntAp–mCherry. Genotoxicity was indicated by the expression of eGFP from the *recAp–egfp* fusion via SOS responses, while the bioavailability of metal ions was indicated by the expression of mCherry from *zntAp–mcherry*. Among the tested materials, only Cr showed a genotoxic effect on *E. coli* above 0.1 mM, even though the other metal ions (Cd, Zn, and Pb) were bioavailable. It was confirmed that Cr is a genotoxic material and the toxic effect of the other metals was caused not via direct DNA damages. Additionally, it was suggested that the WCBs could be applied to investigate the dissolution rates of diverse metallic NPs. Although the dual-sensing WCBs described here were restricted to several metals, it would be applied to diverse metallic NPs if the corresponding metal ion sensing system was available. Of course, further investigation is needed to assess the effects of different concentrations, shapes, and sizes of metallic NPs. Nonetheless, we believe that our study demonstrates the positive prospects of WCBs as a rapid and convenient tool to assess the adverse effects of diverse pollutants.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest to disclose.

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