Toxicology in Vitro 25 (2011) 1132–1142

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Application of cellular biosensors for analysis of bioactivity associated with airborne particulate matter

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ARTICLE INFO

Article history:
Received 4 December 2010
Accepted 25 March 2011
Available online 30 March 2011

Keywords:
Fluorescent Cell Chip
Oxibios
Airborne particulate matter
IL-2
IL-4
IFN-γ
IL-10
NFκB

ABSTRACT

Exposure to airborne particulate matter (PM) is a known risk factor for adverse health effects observed in many environmental and occupational settings. The pathological mechanisms involved in PM-mediated toxicity depend on the size and contents of particles that vary depending on the source of emission. Chemical compositions of PM show multiple components with different bioavailabilities that are capable of acting on multiple molecular and cellular targets, making it difficult to predict PM-associated toxicity based solely on chemical analysis. The aim of the study was to develop robust, sensitive and economical assays for environmental pollutants based on genetically modified mammalian cells. We tested the suitability of two biosensor assays, Fluorescent Cell Chip and Oxibios, developed in part in our laboratories, for assessment of the potential toxicity of airborne PM. Reference PM and PM obtained by sampling of diesel exhaust and indoor air in aluminum and copper facilities in Poland were tested with the two bioassays using unified experimental protocols. Resultant data showed complex patterns of stimulatory and inhibitory activities that were consistent with the origin of PM and might be correlated with their chemical composition. The analysis was informative with regard to type and extent of possible toxicity associated with specific PM and allowed for detection of significant differences between PM from different industrial sites and particular locations within the same industrial sites as well as overall ranking of toxicity risk based on chemical analysis.

1. Introduction

An important part of the routine assessment of the level of air pollution is determination of the amount of airborne particulate matter (PM) (Hauck, 1998). Epidemiological observations strongly support the causal relationship between the exposure to airborne PM and increased mortality and morbidity in exposed populations, mostly due to aggravation of respiratory and cardiovascular diseases (Katsouyanni et al., 2009; Polichetti et al., 2009; Romeo et al., 2006). While the overall amount of PM is an accepted marker of air quality, the risk of particular adverse health effects resulting from exposure to PM is strictly related to the source of emission which determines composition and size of particles (Aust et al., 2002; Chen and Lippmann, 2009; Schwarze et al., 2007). Typically, the chemical speciation of PM reveals organic and elemental carbon, polycyclic aromatic hydrocarbons, metals, transition metals, and other elements (Ntziachristos et al., 2007). It is difficult to link particular components of this complex mixture of organic and inorganic compounds with a specific biological activity of PM. There are examples in which the presence of certain components in PM might be correlated with observed biological activity. For example, the ability of PM to generate reactive oxygen species in exposed cells might be correlated with the presence of transition metals such as iron (Aust et al., 2002), and mutagenicity of PM observed in a standard bacterial assay was correlated with the presence of polycyclic aromatic hydrocarbons (de Aragao Umbuzeiro et al., 2008). While some activities of PM such as the overall redox activity could be directly assessed in a simple assay and attributed to the major components of PM (Ntziachristos et al., 2007), certain more subtle biological effects mediated by PM might result from the presence of a minor amount of a particular component and are difficult to determine in a single assay. Some of the minor components of PM such as lead and mercury (Vassilakos et al., 2007) are known to affect cytokine expression and other functions of immune cells *in vitro* and *in vivo* (Walczak-Drzewiecka et al., 2003; Shen et al., 2001), suggesting possible immunotoxicity that is difficult to determine in a single *in vitro* assay (Carfi et al., 2007). These examples suggest that prediction of various toxic effects mediated

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doi:10.1016/j.tiv.2011.03.019

by PM based solely on chemical analysis might be difficult. Employment of living mammalian cells for testing the bioactivities associated with PM *in vitro* may be useful not only for mechanistic studies but also for routine toxicity testing. In regard to this, there are multiple efforts to develop robust, sensitive and economical assays for environmental pollutants based on different types of biosensors including genetically modified mammalian cells (Pancrazio et al., 1999). We have decided to test the suitability of two cell-based biosensor assays, Fluorescent Cell Chip (Ulleras et al., 2005) and Oxibios, developed in part in our laboratories, for assessment of the potential toxicity of airborne PM. For this purpose, reference PM and PM obtained by sampling of diesel exhaust in The Netherlands and indoor air in two industrial facilities in Poland were tested in parallel with the two bioassays using unified experimental protocols. In both assays we observed reproducible responses that significantly varied in direction and intensity depending on PM origin and correlated with elemental contents of PM samples.

2. Materials and methods

2.1. Chemicals and reagents

Media for cell culture were purchased from Invitrogen, fetal calf serum FCS was obtained from Biowest, geneticin (G418) was purchased from Biochrom AG and hygromycin was purchased from Invivogen. All other chemicals were of analytical grade and obtained from Sigma Chemical Co. unless otherwise stated.

2.2. Collection, analysis and preparation of PM

Standard Reference Material 2584 (trace elements in indoor dust, nominal 1% lead), Standard Reference Material 2585 (organic contaminants in house dust), Standard Reference Material 1648 (urban particulate matter), Standard Reference Material 1649b (urban dust) and Standard Reference Material 1650b (diesel particulate matter) were purchased from the National Institute of Standards and Technology, Gaithersburg, MD, USA. Airborne PM in the aluminum manufacturing plant was collected in two sites, the casting and electrorefining departments, and sieved through a 36-μm mesh strainer. Airborne PM in the copper manufacturing plant was collected in two locations within anode furnace departments (sites A and D) and sieved mechanically through a 25-μm mesh strainer. Diesel particulate matter (DPM) was harvested in the National Institute for Public Health and the Environment (RIVM) in Bilthoven, The Netherlands, using an idling diesel engine (Cassee et al., 2002) and was a kind gift from Dr. Flemming Cassee. The analysis of elements of PM from aluminum and copper plants was performed in the ICP-MS laboratory of the Chemistry Faculty of Warsaw University. In brief, PM samples were dissolved in 63% nitric acid (with hydrofluoric acid addition) and 37% hydrochloric acid followed by mineralization in a closed microwave system. Decomposed samples were diluted with distilled water and analyzed using ICP-MS (inductively coupled plasma mass spectrometry; Elan 6100, Sciex Perkin Elmer) and F-AAS (flame atomic absorption spectrometry; AAnalyst 300, Perkin Elmer). Particle size distribution measurements for PMs were carried out using a laser diffraction instrument Mastersizer (Malvern Instruments Ltd., UK) in National Medicines Institute, Poland. A suspension of 10 mg/ml of PM in distilled water was analyzed before and after 1 h of sonication. Particle size was presented as mean particle diameter $d(0.5)$ (parameter for the particle-size distribution indicating the particle size below which 50% of the volume is present). Prior to experiments with *in vitro* cultured cells, samples of PM were weighed, sterilized in a steam autoclave, suspended in a sterile culture medium and sonicated for 1 h.

2.3. FCC assay

Five EL-4 (mouse lymphoma cell line)-derived reporter cell lines stably transfected with a transgene consisting of promoter regions from mouse β-actin (E/008/009), IL-2 (interleukin-2; E/253/006), IL-4 (interleukin-4; E/452/002), IFN-γ (interferon-γ; E/552/003) and IL-10 (interleukin-10; E/752/005) and ORF (open reading frame) for EGFP (enhanced green fluorescent protein) were described earlier (Ulleras et al., 2005). Reporter cells were cultured at a density of 5×10^5–1×10^6 cells/ml in RPMI 1640 medium supplemented with 2 mM L-glutamine, 20% FCS and 0.5 μg/ml G418 in a humidified atmosphere with 5% CO_2 at 37 °C. The principles of the Fluorescent Cell Chip (FCC) assay were described earlier (Wagner et al., 2006). In brief, cells were seeded at a density of 0.5×10^5 per well in a 96-well plate and incubated with increasing concentrations of tested substance in a total volume of 100 μl at 37 °C in a humidified atmosphere of 5% CO_2 for 18 h. Each plate was arranged to accommodate five reporter cell lines, either activated with PMA (phorbol 12-myristate 13-acetate; 50 ng/ml) and ionomycin (1 μM) or not and exposed to the tested PM suspension at concentrations of 0, 5, 10, 25, 50, 100, 250 and 500 μg/ml. For each cell line there were two controls, one consisting of cells in medium only and another consisting of cells activated with PMA/ionomycin. Following the 18-h incubation, cells were analyzed by flow cytometry to determine the level of EGFP-mediated fluorescence and cell viability.

2.4. Flow cytometry

Before acquisition, the volumes of samples were adjusted to 250 μl with PBS. Cells were analyzed in the Cytomics FC 500 MPL Beckman-Coulter flow cytometer equipped with a multi-plate loader and MXP Acquisition Software v.2.1 (Beckman Coulter Inc.) The level of EGFP-mediated fluorescence of the reporter cells was measured after gating on live cells. In the routine acquisition protocol for determination of EGFP-mediated fluorescence, live cells were selected based on live/dead cell gates on an FS/SS plot. These gates were adjusted and established experimentally by comparing them with the ones set up upon testing a range of immunotoxic substances on EL-4/β-actin reporter cells and staining with dedicated fluorescent dyes: PI (propidium iodide) and 7-AAD (7-aminoactinomycin D). FS/SS gates vs. PI or 7-AAD gates showed good correlation with differences not exceeding ±5% of the analyzed cell population.

2.5. Development of the reporter cell lines for Oxibios assay

For construction of the NFκB-responsive reporter plasmid, an artificial promoter sequence was prepared as an *in vitro*-synthesized DNA oligonucleotide. This promoter sequence contained a 6× concatenated consensus NFκB binding site (5′-GGGAATTTCC-3′) and a minimal core promoter derived from the adenovirus E1b gene. The artificial promoter was cloned into the firefly luciferase-encoding pGL4.14 vector (Promega) and its specific inducibility by NFκB-activating stimuli was verified by transient transfection into several human cancer cell lines. Selection of cell lines for further stable transfection of the construct was based on strength and specificity of induction of transcriptional activity by classical NFκB-activating stimuli (TNFalpha, interleukin-1 and oxidative stress) with corresponding lack of induction by non-cognate signaling pathway activation. Due to presence of expressed receptors and intracellular components of the NFκB pathway, two cancer cell lines of different tissues of origin (ovarian epithelial – SK-OV-3 and hepatocellular – Hep3B) were found to be optimal for specific detection of NFκB activation. The developed vector was used for stable transfection into these cell lines with hygromycin antibiotic selection and clonal

selection by limiting dilution. The SK-OV-3-derived cell lines and the Hep3B-derived ones were maintained in 150 μg/ml hygromycin. The eventual cell line clones IS23-NFkBRE-SK-OV-3 and IS01-NFkBRE-Hep3B were selected to yield the highest luciferase expression enhancement ratio upon treatment with NFκB-activating stimuli.

2.6. Oxibios assay

The NFκB-responsive reporter cell lines were used as cellular biosensors of NFκB activation by PM. Most results were obtained using the SK-OV-3-originated IS23-NFkBRE-SK-OV-3 reporter cell line, which allows for specific and sensitive detection of NFκB activation. In a typical experiment, cells were seeded in 96-well plates at 10^4 cells per well and were incubated without or with various concentrations of dust particles in cell culture medium for 24 h. Subsequently, cells were washed and luciferase reporter gene expression was determined by luminometric assay of luciferase activity in cell lysate (using luciferin as substrate) on the Perkin Elmer EnVision microplate reader. As a direct control for experimental artifacts linked to non-specific dust particle interactions with biosensor components, in some experiments PM was added after 24 h of control incubation in pure cell culture medium and cells were then immediately washed and processed as per standard experimental design. As a control of biosensor functionality, each experiment also included cells exposed to 1 ng/ml TNF and the induction ratio was verified to be stable. In some experiments, 1 ng/ml TNF was added to the cells together with PM to test for synergistic/antagonistic actions.

2.7. Resazurin-based cell viability assay

The resazurin cell viability assay was performed essentially according to O'Brien et al. (2000). Briefly, cells were seeded in 96-well plates at 10^4 per well and exposed to dust particles at varying concentrations for 24 h. Subsequently, cells were washed and resazurin was added to a concentration of 5 μM in culture medium with a further 1-h incubation to allow for resazurin reduction by viable cells. Finally, the fluorescence of reduced resorufin in the medium was determined using the Perkin Elmer EnVision microplate reader at 530 nm excitation and 590 emission wavelengths. Fluorescence intensity is directly proportional to the percentage of remaining viable cells.

2.8. Data presentation and statistical analysis

Experiments were carried out in triplicate and data are presented as the means ± SE. Statistical significance of observed differences was tested with ANOVA-followed by a Holm–Sidak test using SigmaStat 2.03 software (SPSS Inc., Chicago, IL, USA).

The immunotoxic effect score values for particulate matter bioactivity measured by the FCC biosensors were derived from statistically significant changes in the level of EGFP in each reported cell lines that exceeded control by 20%. Increase in cytokine reporter cell lines either resting or activated was scored 1 per each occurrence. Concomitant increase observed in the same cytokine reporter cell line under both resting and activated condition was additionally scored 1 per each occurrence. Decrease in activated cytokine reporter cell line was scored 1 per each occurrence. Decrease in activated cytokine reporter cell line that occurred without concomitant decrease in control β-actin reporter cell lines was additionally scored 1 per each occurrence.

The scoring procedure for Oxibios biosensor was based on viability-adjusted deviations from control NF-kappaB activity values. Specifically, the absolute difference between percentage of activation and percentage of cell viability was rounded down to the nearest 10% and divided by 10 for each particulate matter concentration used. Subsequently, these partial scores were weighted (multiplied by 2 for concentrations below 100 μg/ml, divided by 2 for values not statistically significant in the Holm–Sidak post hoc test) and their sum was adopted as the final toxic effect score. The weighting was performed in order to enhance the status of particulate matter types which showed significant interference with the NF-kappaB pathway even at low concentrations of exposure.

Toxic elements content index of given particulate matter was calculated by summing contents of ten elements with established Minimal Risk Levels (MRLs) (http://www.atsdr.cdc.gov/mrls/mrllist.asp), namely: Al, As, Ba, Be, Cd, Co, Cu, Sr, V and Zn divided by their respective MRLs (1000.0, 0.3, 200.0, 2.0, 0.1, 10.0, 10.0, 2000.0, 10.0 and 30.0 μg/kg/day).

Spearman's rank correlation coefficient was calculated between the overall toxicity rank and the toxic element content rank and its significance was tested using SigmaStat 2.03 software.

3. Results

3.1. Characteristics of tested PM samples

Two sets of PM samples were employed in a series of experiments for assessment of associated bioactivity using two cellular biosensor-based assays. The reference PMs (SRM No. 1648, 1649b, 1650b, 2584 and 2585) are extensively characterized (Canepari et al., 2006; Bamford et al., 2003; Murphy et al., 2000; Stapleton et al., 2006) and were previously frequently employed as reference samples for *in vitro* toxicological studies (Karlsson et al., 2004; Singh et al., 2005; Auten et al., 2009; Braun et al., 2010; Mo et al., 2009; Sawyer et al., 2010). Their elemental composition is compiled in Table 1. The chemical composition of the PM sample obtained from diesel engine exhaust was previously reported (Cassee et al., 2002) and its elemental content was summarized in Table 2. The PM samples collected at two industrial facilities in Poland were analyzed for their elemental content in the present study. The analysis (Table 2) demonstrated the presence of multiple elements including cadmium, cobalt, lead, strontium, aluminum, copper, zinc, arsenic and bismuth, known to mediate different toxic effects in human and animals (Prozialeck et al., 2008; Nemery, 1990; Clarkson, 1987).

3.2. PM bioactivity screening using FCC biosensors

We first applied biosensor-based assays for immunotoxicity screening, using the FCC assay (Ulleras et al., 2005; Wagner et al., 2006) to test reference PM samples. To this end, a panel of reporter cell lines was exposed to increasing concentrations of reference PM suspension with or without additional activation with PMA and ionomycin. As seen in Fig. 1A–E, following an 18-h incubation, all reference PM suspensions resulted in a statistically significant increase in the level of EGFP fluorescence in selected reporter cell lines. The strongest and dose-dependent induction was observed in the IL-2 reporter cell line exposed to SRM 1648 (Fig. 1A) followed by 1649b (Fig. 1B), 2584 (Fig. 1D) and 2585 (Fig. 1E). SRM 1648 and 2584 resulted in increased EGFP expression in the IL-2 reporter cell line activated with PMA and ionomycin (Fig. 1F and I), and SRM 1648 resulted in increased fluorescence in the reporter line for IL-4 with and without additional activation with PMA and ionomycin (Fig. 1A and F). There was also a small decrease in fluorescence following exposure of activated reporter cells to SRM 2585 (IL-4 and IL-10) and 2584 (IL-4). A small (not exceeding 8%) decrease in cell viability of reporter cells was observed for high concentrations of SRM 1648, 1649b and 1650b, and a slightly greater decrease in cell viability (15%) was observed for high

Table 1
Comparison between levels of major and trace elements in particulate matters of standard reference materials.

Material	SRM 1648	SRM 1649b	SRM 1650b	SRM 2584	SRM 2585
Mean particle diameter $d(0.5)$[a]	5.85 μm	24.4 μm	0.18 μm	7.75 μm[b]	26.54 μm[b]
Element	Mass fraction in μg/g ± SD				
Al	34 300 ± 1300	3.74 ± 0.93[c]	<dl[d]	23 200 ± 600	–[e]
As	115.5 ± 3.9	67 ± 2	3[d]	17.4 ± 4.2	10.12[f]
Ba	737 ± 2.74[c]	569 ± 21	–	1300	–
Be	3.08 ± 4.62[c]	2.55 ± 5	–	0.7	–
Bi	10.5 ± 8.21[c]	6.57 ± 6.85[c]	–	9	–
Cd	73.7 ± 2.3	22	<dl[d]	10.0 ± 1.1	8.11[f]
Co	17.93 ± 0.68	16.4 ± 0.4	–	10	–
Cu	610 ± 70	223 ± 7	50[d]	320	–
Ga	15.4 ± 10.3[c]	16.9 ± 1.24[c]	–	6.4	–
Li	14.1 ± 8.2[c]	13.8 ± 15.3[c]	–	17	–
Mn	790 ± 44	237 ± 8	15[d]	370	–
Mo	17.6 ± 3.92	13.5 ± 0.9	–	5.5	–
Pb	6550 ± 330	12 400 ± 440	23[d]	9761 ± 67	2988[f]
Sc	5.15 ± 17.9[c]	8.7 ± 0.2	–	4	–
Sr	215 ± 17	157 ± 0.65[c]	<dl[d]	160	–
Tb	–	–	–	–	–
U	5.53 ± 2.9[c]	2.65 ± 0.08	–	1.6	–
V	127 ± 11	345 ± 13	<dl[d]	34	–
Zn	4800 ± 270	1680 ± 40	870[d]	2580 ± 150	–
Y	14.3 ± 6.55[c]	16.3 ± 5.96	–	10	–

Concentration values of elements and particle size characteristics of SRM 1648, SRM 1649b, SRM 2583 and SRM 2584 were obtained from certificates of analysis of standard reference materials provided by National Institute of Standards and Technology (http://www.nist.gov/index.html) unless otherwise stated.
<dl – below detection limits.
[a] $d(0.5)$ indicates the particles size below which 50% of the volume is present.
[b] Particle size measured as described in Section 2, after 1 h sonication in water.
[c] Element concentration measured by Pineiro-Iglesias et al. (2003).
[d] Element concentration measured by Huggins et al. (2000).
[e] No value reported.
[f] SRM 2585 consists of 70% of SRM 2583 and 30% of SRM 2584. Elements content in SRM 2585 was estimated on the basis of element contribution of constituent SRMs in the SRM 2585 blend. Number of presented elements is limited by data availability for SRM 2583.

Table 2
Comparison between levels of major and trace elements in smelter airborne particulate matters and diesel engine exhaust particulate matter.

Material	Aluminum casting dept.	Aluminum electrorefining dept.	Copper anode furnace A	Copper anode furnace D	Diesel PM
Mean particle diameter $d(0.5)$[a]	25.19 μm/–[b]	26.29 μm/8.65μm[b]	13.77 μm/10.28μm[b]	15.22 μm/9.77μm[b]	4.2 μm/1.8μm[b]
Element	Mass fraction, μg/g ± SD				
Al	50 000	30 000	5000	20 000	63.53
As	14.3 ± 1.2	1.99 ± 0.18	177 ± 6	7.66 ± 0.31	0.465
Ba	1024 ± 1	2.29 ± 0.15	32.8 ± 0.2	5.48 ± 0.35	17.06
Be	13.6 ± 0.6	0.56 ± 0.13	1.28 ± 0.06	2.14 ± 0.26	0.005
Bi	0.336 ± 0.116	0.298 ± 0.08	10.6 ± 0.2	0.318 ± 0.004	0.046
Cd	7.45 ± 0.36	0.78 ± 0.06	21.1 ± 0.2	0.32 ± 0.07	0.185
Co	2.02 ± 0.06	1.15 ± 0.03	27.3 ± 0.1	8.55 ± 0.09	0.141
Cu	782 ± 13	9 ± 0.3	90 000	123 ± 1	4.99
Ga	9.71 ± 0.23	31.9 ± 0.23	3.233 ± 0.001	18.5 ± 0.05	0.011
Li	77.6 ± 0.6	10.2 ± 1.9	17.1 ± 0.5	388 ± 6	0.145
Mn	58.0 ± 1.4	2.67 ± 0.21	108 ± 1	117 ± 1	5.74
Mo	3.94 ± 0.44	0.8 ± 0.06	30.59 ± 0.03	1.17 ± 0.16	0.42
Pb	25 ± 0.4	12.7 ± 0.4	169 ± 5	33.3 ± 0.9	9.6
Sc	1.14 ± 0.24	0.358 ± 0.028	1.89 ± 0.13	8.29 ± 0.15	0.014
Sr	21.5 ± 0.4	2.46 ± 0.16	10.7 ± 0.1	90.5 ± 1.3	1.05
Tb	0.116 ± 0.021	0.024 ± 0.004	0.375 ± 0.159	0.26 ± 0.024	0.001
U	0.485 ± 0.041	0.061 ± 0.004	0.234 ± 0.002	2.55 ± 0.06	0.006
V	20.4 ± 0.7	26.4 ± 1.4	16.3 ± 0.8	54.5 ± 0.1	1.95
Zn	277 ± 7	61 ± 0.1	884 ± 7	962 ± 3	57.53
Y	1.1 ± 0.01	0.041 ± 0.024	1.32 ± 0.14	5.65 ± 0.4	0.04

[a] $d(0.5)$ indicates the particles size below which 50% of the volume is present.
[b] Particle size before/after 1 h of sonication in water.

concentrations of SRM 2584 and 2585 (Fig. 1A–J). We next performed FCC testing of a set of PM obtained by sampling diesel exhaust and air at different industrial locations. As seen in Fig. 2A–J, some of these samples revealed significant activity. Incubation with PM from an electrorefining department in an aluminum smelter resulted in increased fluorescence in reporter cells for IL-4 and IL-2 and to a much lesser degree, IL-10 and β-actin (Fig. 2B). Incubation with PM from site A in a copper plant resulted in a significant increase in fluorescence of reporter cells for IL-4 and IL-10 associated with a large decrease in cell viability (Fig. 2C). Exposure to DPM resulted in a small but significant increase in fluorescence in reporter cells for IL-2, IL-4 and IFN-γ

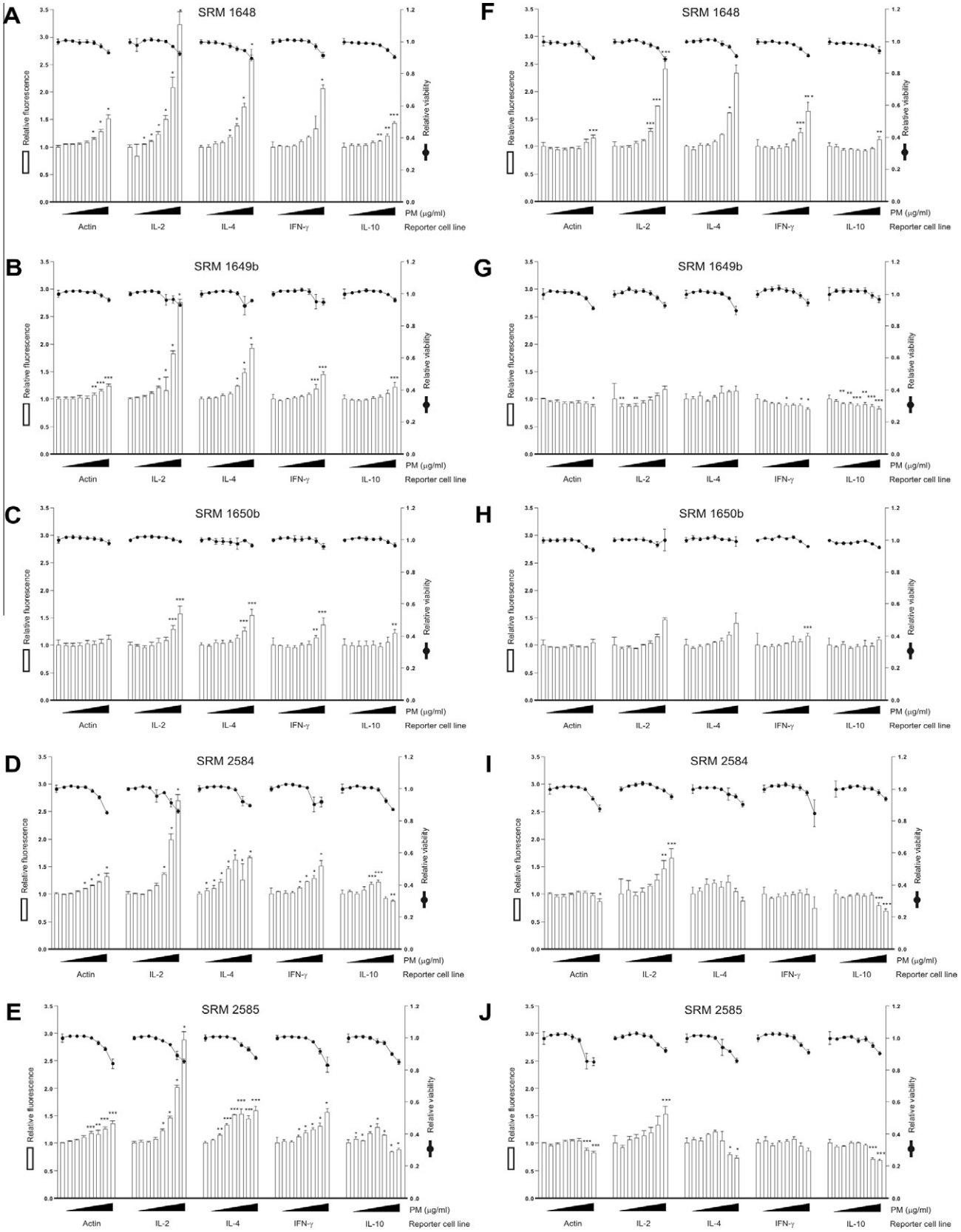

Fig. 1. The fluorescence and viability patterns obtained with the FCC test for standard reference materials. Five reporter cell lines for β-actin, IL-2, IL-4, IFN-γ and IL-10 were exposed to a series of dilutions of tested dust particles (0, 5, 10, 25, 50, 100, 250 and 500 μg/ml). Reporter cells were either resting (A–E) or activated with PMA/ionomycin (F–J). Mean intensity of fluorescence (bars, left *Y*-axis) and cell viability (points, right *Y*-axis) were determined for each experimental condition using flow cytometry and normalized against control (medium only) for resting and activated control (PMA/ionomycin) for activated. Each data point represents the mean ± SEM from three independent experiments. Significance of differences between means: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control.

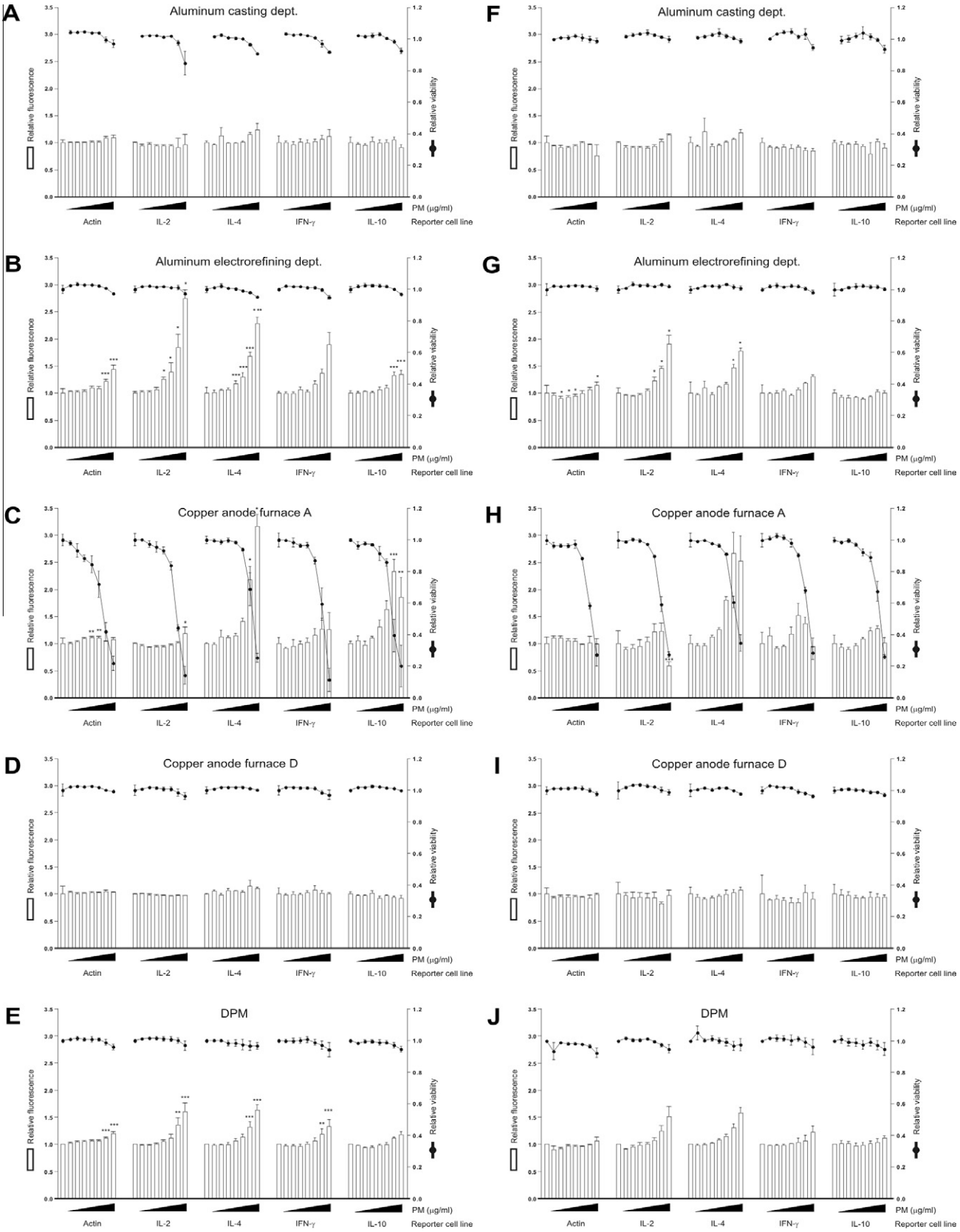

Fig. 2. The fluorescence and viability patterns obtained with the FCC test for airborne PM from aluminum and copper plants and DPM. Five reporter cell lines for β-actin, IL-2, IL-4, IFN-γ and IL-10 were exposed to a series of dilutions of tested dust particles (0, 5, 10, 25, 50, 100, 250 and 500 µg/ml). Reporter cells were either resting (A–E) or activated with PMA/ionomycin (F–J). Mean intensity of fluorescence (bars, left *Y*-axis) and cell viability (points, right *Y*-axis) were determined for each experimental condition using flow cytometry and normalized against control (medium only) for resting and activated control (PMA/ionomycin) for activated. Each data point represents the mean ± SEM from three independent experiments. Significance of differences between means: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control.

(Fig. 2E). In reporter cells additionally activated with PMA and ionomycin, the only statistically significant effects were observed for PM from the electrorefining site in an aluminum smelter, resulting in increased fluorescence in reporter cells for IL-2, IL-4 and β-actin (Fig. 2G). Other PM samples did not result in changes in fluorescence in resting and activated reporter cell lines. Thus, in the FCC test, cytokine-specific reporter cell lines exposed to different PM suspensions showed different patterns of changes in reporter gene expression.

3.3. PM bioactivity screening using Oxibios biosensors

First, in a series of experiments we compared reporter cell lines IS01-NFkBRE-Hep3B originating from Hep3B hepatocytes and IS23-NFkBRE-SK-OV-3 originating from SK-OV epithelial cells. We observed significant differences in the magnitude of TNF-mediated luciferase activity with IS23-NFkBRE-SK-OV-3 responding to a much greater extent compared to IS01-NFkBRE-Hep3B cells (data not shown). Similarly, the preliminary experiments showed a higher and more reproducible response of IS23-NFkBRE-SK-OV-3 reporter cells to reference PM as compared to IS01-NFkBRE-Hep3B. Thus, we next employed IS23-NFkBRE-SK-OV-3 reporter cells for systematic analysis of bioactivities associated with PM. As seen in Fig. 3A, reference PM SRM 1648, 2584 and 2585 resulted in statistically significant and dose-dependent increases in luciferase activity in IS23-NFkBRE-SK-OV-3 cells. In contrast, SRM 1650b resulted in a significant and dose-dependent decrease in luciferase activity observed both in resting and TNF-activated IS23-NFkBRE-SK-OV-3 reporter cells (Fig. 3A and B). Incubation of reporter cells with SRM 1648 resulted in significant cytotoxicity (Fig. 3C). We next performed testing with IS23-NFkBRE-SK-OV-3 reporter cells with another set of PM from diesel exhaust and from air at different industrial locations. As seen in Fig. 4A, PM from both locations in the aluminum manufacturing plant resulted in an increase and PM from copper anode furnace A and to a lesser degree PM from diesel exhaust resulted in a decrease in luciferase activity. The significant inhibition of luciferase activity with PM from copper anode furnace A and diesel exhaust was observed not only in resting cells but also reporter cells activated with TNF (Fig. 4B). While the inhibition of luciferase activity by PM from copper anode furnace A was associated with strong cytotoxicity, the PM from diesel exhaust did not cause a significant decrease in cell viability (Fig. 4C).

3.4. Bioactivity ranking of tested PM samples

Next we employed a scoring system described in Section 2 to compare the bioactivities of different PM samples observed in two different biosensors based tests. As seen in Table 3 scores for

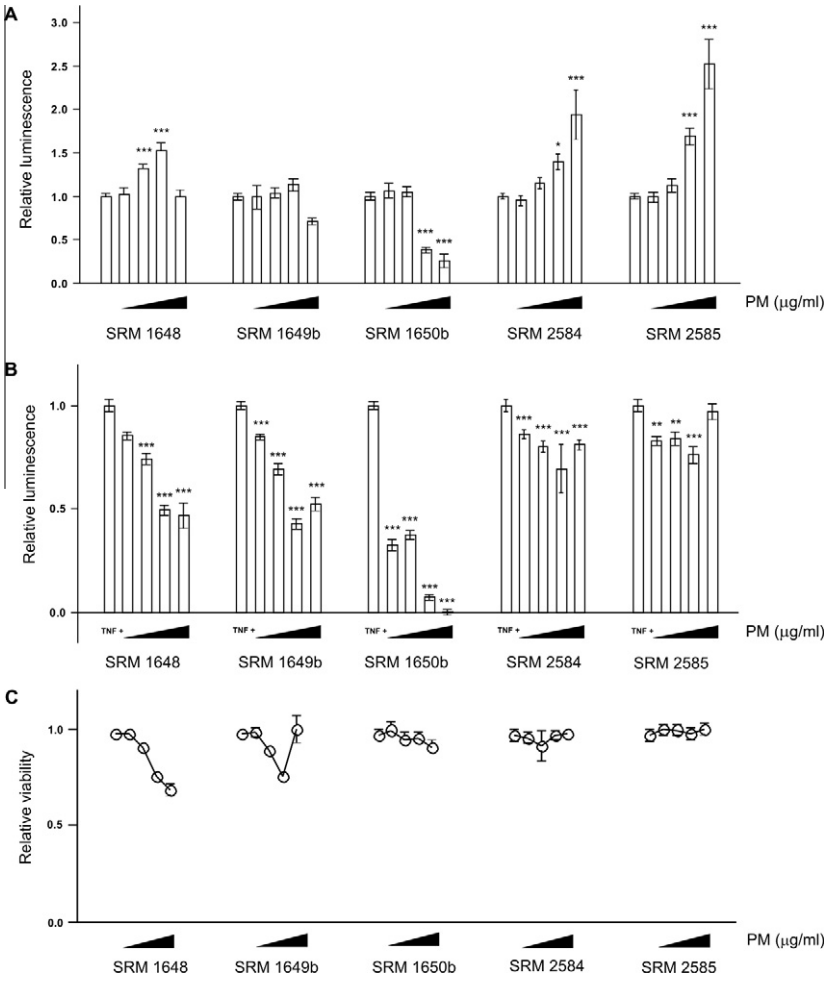

Fig. 3. The luminescence and viability patterns obtained with SK-OV-3-derived NFκB-responsive reporter cell line for standard reference materials. Reporter cells were exposed to a series of dilutions of tested dust particles (0, 10, 50, 200 and 500 μg/ml). Cells were either resting (A) or TNF-activated (B). Luminescence determined for each experimental condition were normalized against control (medium only) for resting and activated control (TNF) for activated. Viability of resting reporter cells treated with dust particles was normalized against control-untreated cells (C). Each data point represents the mean ± SEM from three independent experiments. Significance of differences between means: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control.

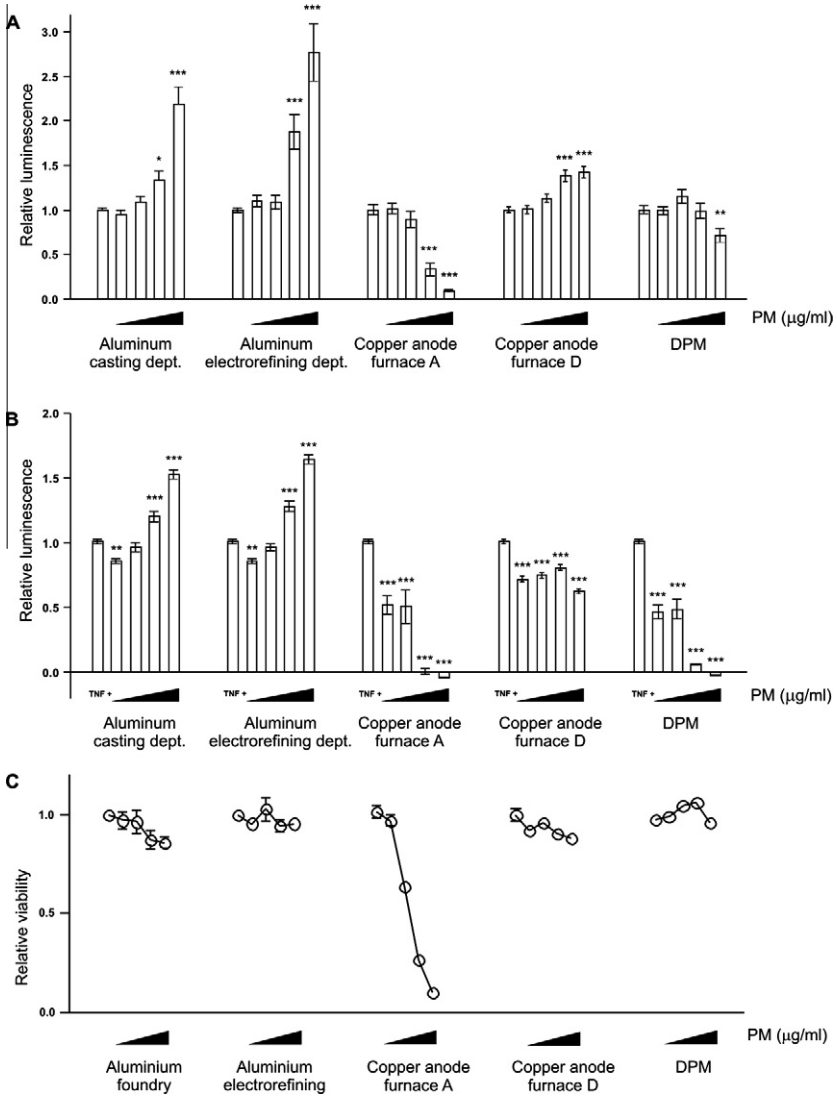

Fig. 4. The luminescence and viability patterns obtained with SK-OV-3-derived NFκB-responsive reporter cell line for airborne PM from aluminum and copper plants and DPM. Reporter cells were exposed to a series of dilutions of tested dust particles (0, 10, 50, 200 and 500 μg/ml). Cells were either resting (A) or TNF-activated (B). Luminescence determined for each experimental condition were normalized against control (medium only) for resting and activated control (TNF) for activated. Viability of resting reporter cells treated with dust particles was normalized against control-untreated cells (C). Each data point represents the mean ± SEM from three independent experiments. Significance of differences between means: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control.

Table 3
Rankings of tested airborne particle matters based on toxic bioactivity and content of selected toxic elements.

	FCC ranking /(score)	Oxibios ranking /(score)	Biosensors ranking /(score)	Toxic elements ranking /(score)
Aluminum electrorefining dept.	2/(19.0)	1/(28.0)	1/(47.0)	3/(30.01)
SRM 2585	3–4/(13.0)	2/(23.0)	2/(36.0)	n. d.
SRM 1648	1/(21.0)	5/(14.0)	3/(35.0)	2/(34.89)
SRM 2584	3–4/(13.0)	4/(15.0)	4/(28.0)	4/(23.78)
SRM 1650b	6–7/(6.0)	6/(12.0)	5–6/(18.0)	n. d.
Aluminum casting dept.	8–10/(0.0)	3/(18.0)	5–6/(18.0)	1/(50.26)
SRM 1649b	5/(8.0)	8/(3.5)	7/(11.5)	7/(0.44)
Copper anode furnace D	8–10/(0.0)	7/(10.0)	8/(10.0)	5/(20.18)
Diesel PM	6–7/(6.0)	9/(3.0)	9/(9.0)	8/(0.07)
Copper anode furnace A	8–10/(0.0)	10/(2.0)	10/(2.0)	6/(5.93)

Score values were calculated as described in Section 2 and used to rank PM samples from the most to the least bioactive ones.
n. d. – indicates sample not included into ranking due to the lack of data on its elemental composition.

each PM obtained for individual tests were added to calculate the combined score as a measurement of a general level of toxicity risk related bioactivities associated with given PM sample. This allowed us to perform ranking of tested PMs with regard to their responses in biosensor-based tests (both in each test separately and combined) in order to facilitate drawing conclusions from our complex quantitative data. We have also calculated a score for content of ten toxic elements with established MRLs (Table 3) and analyzed

the correlation between combined biosensors activity and toxic element content and found significant positive correlation (Spearman's rank correlation coefficient = 0.714, $p = 0.0374$).

4. Discussion

4.1. Preliminary assessment of PMs toxicity could be performed with a set of in vitro cellular tests

The assessment of the risk of toxicity associated with a complex and unstable mixture of multiple compounds found in PM presents a difficult task because of parallel interactions of multiple potentially toxic compounds delivered to living cells; these compounds have different bioavailabilities related to different solubilities and sizes of particles. In addition to increasing knowledge of the chemical composition of PM originating from different sources, a development of a novel, fast and robust method of direct detection of toxicity *in vitro* could improve our ability to predict such a risk. One possible method is employment of living cells for *in vitro* exposure to PM. Cytotoxicity is one obvious endpoint of such assays that has been employed for testing PM-associated toxicity *in vitro* (Frampton et al., 1999; Hsiao et al., 2000; Muller et al., 2006). However, extrapolation of data from a cytotoxicity assay into expected *in vivo* effects is limited mostly to acute toxicity (Shrivastava et al., 1992; Dierickx, 2000). More challenging is usage of *in vitro* testing for prediction of chronic toxicity including organ, system and developmental stage specific toxic effects of exposure such as hepatotoxicity, immunotoxicity and developmental toxicity to name only few. Frequently it has been proposed that the entire battery of *in vitro* tests rather than a single *in vitro* test should be employed for such purpose (Spielmann, 2009). We have used two different cellular biosensor based tests, FCC previously prevalidated with a set of 63 compounds for immunotoxicity testing (Wagner et al., 2006) and Oxibios detecting xenobiotics effects on NFκB signaling pathways, for assessment of potential toxicity of several PM samples. While the phenotypes of cells employed for testing do not resemble the phenotype of cells coming into direct contact with PM *in vivo*, these tests allow for detection of toxic influences of tested samples at the subcellular (signaling pathway) level as reporter cell lines were selected and optimized for the detection of modulatory effects of xenobiotics.

4.2. Cellular biosensors detected bioactivities of PMs that are consistent with toxic effects of PMs observed in vivo

The FCC assay showed that most of the tested PM mediated statistically significant effects suggesting potential immune activation. There were visible similarity of patterns generated by FCC assay for two indoor dust reference samples (SRM 2584 and 2585) (Fig. 1D and E) and two reference PM samples representing ambient urban air (SRM 1648 and 1649B) (Fig. 1A and B). Our assays did not reveal significant differences in bioactivities between reference PM from indoor dust contaminated with Pb and organics. The observations that PM obtained from indoor dust and urban air demonstrated activatory effects is consistent with observations in animal allergic sensitization models demonstrating adjuvant activity of ambient PM obtained from indoor dust and urban air (Steerenberg et al., 2004; Ormstad, 2000) and with epidemiological data linking such exposure to increased incidents of allergic sensitization (Brauer et al., 2007; Parker et al., 2009). Activities of PM from different indoor dust and urban air reference samples observed in the Oxibios assay were also similar to each other. SRM 1648 and 1649b mediated significant inhibition of TNF-mediated NFκB activation associated with significant cytotoxicity, and SRM 2584 and SRM 2585 mediated weak inhibition of TNF-mediated NFκB activity and significant activation of NFκB in resting cells without noticeable cytotoxicity (Fig. 3). There was also visible similarity in the pattern of bioactivities mediated in both assays by two different samples of PM from diesel exhaust. In the FCC assay, both reference sample SRM1650b (Fig. 1C) and the sample obtained from RIVM (Fig. 2E) mediated increased fluorescence in reporter cells for IL-2, IL-4 and IFN-γ. In the NFκB assay, both of these samples mediated strong inhibition of resting and TNF-stimulated NFκB activity without a noticeable decrease in cell viability. Observation of stimulation of cytokine promoter activity in the FCC assay (Figs. 1C and 2E) is consistent with data showing that this type of PM stimulated expression of IFN-γ in human dendritic cells and exhibited adjuvant activity in an animal model of allergic sensitization (Muranaka et al., 1986; Steerenberg et al., 2004). The inhibitory activity on NFκB observed in the Oxibios assay (Figs. 3 and 4) seems to be in disagreement with reports suggesting that diesel exhaust particles in exposed cells induced oxidative stress, presumably activating the NFκB pathway (Sawyer et al., 2010; Baeza-Squiban et al., 1999). It is worth mentioning that there are reports suggesting inhibition of NFκB activity by another air pollutant, tobacco smoke, by upregulation of HSP70 (Vayssier et al., 1998), and that upregulation of HSP70 was also observed in epithelial cells exposed to diesel exhaust extracts (Jung et al., 2007). Thus, several discrete observations of particular bioactivities obtained during testing of PM samples with FCC and Oxibios was consistent with reported effects similar PM in other *in vitro* and *in vivo* model as well as epidemiological observations.

4.3. Bioactivities of PMs detected by cellular biosensors could be linked to particular chemical components of PMs

Chemical speciation of PM obtained in aluminum and copper processing plants showed (Table 2) the presence of multiple metals and nonmetals, including cadmium, cobalt, lead, strontium and arsenic, that are known toxicants (Prozialeck et al., 2008; Nemery, 1990; Clarkson, 1987) and have been reported to mediate some of the toxic effects of PM observed *in vitro* and *in vivo* (Park et al., 2007; Molinelli et al., 2002; Schaumann et al., 2004; Chen and Lippmann, 2009). The biosensor-based assays showed significant differences in PM-mediated effects depending on the particular site of air sampling. While PM obtained in the aluminum casting department did not demonstrate significant activity except for decreased cell viability at the highest PM concentrations, the PM from the aluminum electrorefining department mediated significant activation of reporter cell lines in the FCC assay suggesting that this PM sample could upregulate IL-2 and IL-4 expression in lymphocytes and mediate immunotoxicity (Wagner et al., 2006). Observed differences in activities of these two PM in the FCC test might be related to the respective differences in their elemental composition, with PM from the casting department containing greater amounts of As, Cd, Pb and Sr, which could explain the observed cytotoxicity (Table 2). Interestingly, the more active PM from the electrorefining department contained over 3-fold more of the known immunotoxicant Ga as compared to the other sample from the aluminum plant; however, known effects of Ga compounds on the immune system are predominantly suppressive and do not include upregulation of cytokine production in lymphocytes (Lewis et al., 1996; Gondre-Lewis et al., 2003). One possible explanation of the high activity of PM from the aluminum electrorefining department might be the expected presence of organic compounds, such as polycyclic aromatic hydrocarbons (PAH), emitted during electrolytic processes (Brzeznicki and Przybylski, 1996; Vanrooij et al., 1992) and PM from diesel exhaust another PM known to contain PAH (Bamford et al., 2003) has also mediated preferential activation of IL-2 and IL-4 reporter cells in FCC tests. In the Oxibios biosensor-based assay, two PMs from aluminum plant

showed similar activity suggesting a capability of activating NFκB that could be explained by the presence in both samples of Cd, Pb and As (Table 2), elements known to activate the NFκB pathway in different types of cells *in vitro* (Misra et al., 2002; Huang et al., 2001; Zuscik et al., 2007). Samples from two sites in a copper plant (site A and site D) also showed significant differences in the FCC assay with PM from site A causing significant cytotoxicity in all reporter cell lines associated with increased fluorescence in resting and activated IL-4 reporter cell line and PM from site D being practically inert. These differences might be explained by different content of PM; the sample from site A contained several hundred times more Cu, and several fold more As, Bi, Cd, Co, Mo and Pb as compared to the sample from site D (Table 2). Assuming that one tenth of the content of Cu was soluble and accessible for reporter cells, the concentration of Cu ions at the highest tested concentration of PM from site A could reach 7×10^{-5} M, a concentration at which Cu salts mediated significant cytotoxicity (Schmalz et al., 1998). Two PM samples from the copper plant also demonstrated a different pattern of activities in the Oxibios assay, with PM from site A exhibiting inhibitory activity possibly related to significant cytotoxicity that was also observed in the FCC test and PM from site D mediating a small increase in NFκB activity in resting cells (Fig. 4A) combined with modest inhibition of TNF-mediated NFκB activity (Fig. 4B). Considering the known mechanism of NFκB activation, it is possible that the complex pattern of activatory and inhibitory effects mediated by this sample could be explained by the presence of multiple NFκB activators such as Cd, Pb and As (Misra et al., 2002; Huang et al., 2001; Zuscik et al., 2007) and relatively high amounts of Zn (Table 2), which is known to inhibit NFκB signaling *in vitro* and *in vivo* (Ho et al., 2001; Szuster-Ciesielska et al., 2009). Thus, several observations obtained during testing of PM samples with FCC and Oxibios could be linked to the presence in these PM samples particular toxic elements.

4.4. The level of bioactivities of PM detected by cellular biosensors positively correlates with PM content of toxic elements

To measure the general level of toxicity risk related bioactivities associated with different PM samples in set of biosensor-based tests we employed a scoring system (Table 3) and rank analyzed PMs according to the relative level of biosensors responses. Interestingly this ranking strongly positively correlated with the presence of toxic elements with established MLRs values in PM samples (Table 3). This suggests that the differences in the patterns of bioactivities detected by biosensors might be at least in part explained by the respective differences in the contents of several toxic elements such as Al, As, Ba, Be, Cd, Co, Cu, Sr, V and Zn.

5. Conclusion

The use of cellular biosensors for testing PM *in vitro* allowed us to detect significant differences in their bioactivities that could be used for the preliminary assessment of PM-mediated toxicity. The pattern of PM-mediated bioactivities was consistent with the origin of PM, and different samples of PM from urban air, indoor dust, and diesel exhaust showed a high degree of similarity in both biosensor-based assays. At the same time, this approach allowed for the detection of significant differences in bioactivities between PM from different industrial sites and different locations within the same industrial sites that significantly differed in their chemical composition. Specifically, aluminum plants were judged to be workplaces associated with higher occupational hazards due to immunomodulatory and oxidative toxicity than copper plants, which correlated well with their toxic elements content. While a significant part of biosensor-detected toxic effects seems to be explainable by inorganic pollutant content in the PM, the organic toxicants contained in them might be much harder to determine are probably also responsible for some differences in cellular biosensor detected toxicity risk related activity. This underscores the potential applicability and relevance of *in vitro* toxicity testing by cell-based biosensors for routine assessment of health risks which would be difficult to discover by purely chemical analysis.

Conflict of interests

J. Dastych is a shareholder of the company that possesses part of the rights to the patent protecting commercial application of the FCC test.

Acknowledgments

This study was supported in part by the Grant PL0107 "Cellular biosensors for automated monitoring of environmental pollution" from the Norwegian Financial Mechanism and EEA Financial Mechanism and the Polish Ministry of Science and Higher Education and in part by Grants 2 P04B 00129 and PB3459/B/P01/2007/33 from the Polish Ministry of Science and Higher Education.

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