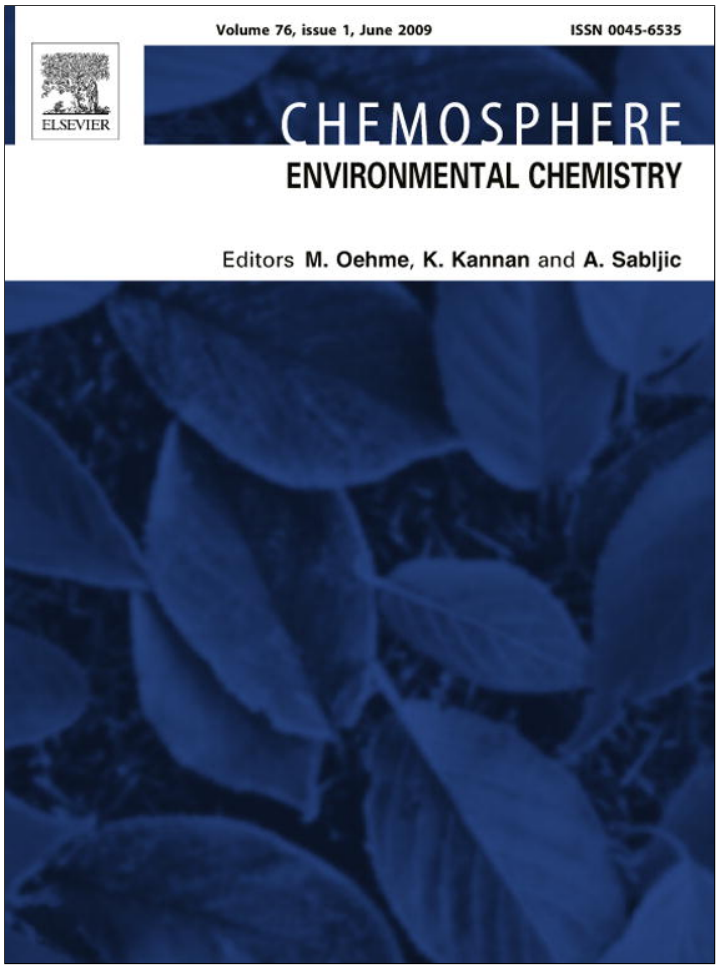

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Development of a dynamic delivery method for in vitro bioassays

Jung-Hwan Kwon [a,b,*], Thomas Wuethrich [a], Philipp Mayer [c], Beate I. Escher [a,d]

[a] Department of Environmental Toxicology (Utox), Swiss Federal Institute of Aquatic Science and Technology (Eawag), Überlandstrasse 133, P.O. Box 611, 8600 Dübendorf, Switzerland
[b] Department of Environmental Engineering, Ajou University, Woncheon-dong, Yeongtong-gu, Suwon 443-749, Republic of Korea
[c] National Environmental Research Institute, Aarhus University, Frederiksborgvej 399, DK-4000 Roskilde, Denmark
[d] National Research Centre for Environmental Toxicology (EnTox), University of Queensland, 39 Kessels Rd., Brisbane, Qld 4108, Australia

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ABSTRACT

Measuring the biological activity of hydrophobic chemicals using in vitro assays is challenging because their aqueous solubility is low and the high density of bio-suspensions strongly decreases the bioavailability of hydrophobic pollutants. Dynamic dosing by partitioning from a stable polymer has a potential to overcome these limitations. Poly(dimethylsiloxane) (PDMS) was chosen due to its documented bio-compatibility and excellent partitioning properties. PDMS sheets were loaded with five polycyclic aromatic hydrocarbons (PAHs) and then immersed in model bio-suspensions composed of membrane vesicles ("chromatophores", composed of 30% lipids and 70% proteins) isolated from the photosynthetic bacterium *Rhodobacter sphaeroides* or phospholipid bilayer vesicles (liposomes) composed of palmitoyl-oleoyl phosphatidylcholine (POPC). Method development included the determination of partition coefficients between chromatophores or liposomes and water, desorption rate constants from PDMS to bio-suspensions, and diffusion resistances in both PDMS and bio-suspensions. The release of the PAHs from the PDMS into the bio-suspensions was measured and modeled as a combination of diffusion in pure water and diffusion in a completely mixed solvent composed of water and bio-suspensions. The mass transfer resistance for the release was lower in the PDMS than in the tested solutions, which demonstrates that PDMS can efficiently deliver PAHs even to dense biosuspensions. The contribution of aqueous diffusion to the mass transfer decreased with increasing hydrophobicity of the PAHs indicating that hydrophobic chemicals are efficiently transported with suspended biomaterial. The passive dosing system is versatile and offers a number of applications. Promising are tests with instantaneous response, where the time-dependent effect can be translated to concentration-effect curves but the system is also applicable for assuring constant dosing for longer-term testing.

1. Introduction

In vitro bioassays are promising tools for screening of biological activity of chemical pollutants but they often lack sensitivity as compared to in-vivo testing (e.g., Schirmer et al., 2008). Chemicals are typically administered in the assay medium by dissolving them directly into the solution or by adding them as dissolved in co-solvents. Depending on the chemical properties, it may become challenging to maintain constant exposure condition in the assay medium during the course of the experiment. For example, volatile compounds can be lost during the experiment and lipophilic chemicals can bind onto the plastic surfaces of the plate and into cellular matrices (Schirmer et al., 1997; Hestermann et al., 2000; Heringa et al., 2004; Gülden and Seibert, 2005; Schreiber et al., 2008). In addition, in many cell-based bioassays, the presence of large amounts of serum protein is necessary, which has a high sorptive capacity for hydrophobic chemicals and therefore decreases the bioavailable concentration in a given assay system (Heringa et al., 2003; de Bruyn and Gobas, 2007). Although solubility of hydrophobic chemicals may be enhanced by co-solvents (Bendels et al., 2006), the presence of co-solvents may interfere with biological activity by changing the conformation and mobility of proteins (Makhatadze and Privalov, 1995; Fadnavis et al., 2005). Therefore, it would be ideal to introduce chemicals into the assay medium without using co-solvents.

Partition controlled delivery (partitioning driven administration or passive dosing) is a new experimental technique without organic co-solvents (Mayer et al., 1999; Brown et al., 2001; Mayer and Holmstrup, 2008). Stable polymers such as poly(dimethylsiloxane) (PDMS), containing hydrophobic organic chemicals, can be placed in the assay medium and compounds are passively delivered from PDMS to the assay medium by thermodynamic partitioning. Because partitioning processes between solution and dissolved

* Corresponding author. Address: Department of Environmental Engineering, Ajou University, Woncheon-dong, Yeongtong-gu, Suwon 443-749, Republic of Korea. Tel.: +82 31 219 1942; fax: +82 31 215 5145.
E-mail address: jhkwon@ajou.ac.kr (J.-H. Kwon).

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humic acids (e.g., Schlautmann and Morgan, 1993) or liposomes (e.g., Cócera et al., 2001) are known to be extremely fast, it is reasonable to assume that the diffusion in the aqueous boundary layer at the polymer–solution interface is the rate-limiting step of the overall dosing process. Using this passive dosing technique, different concentrations for a dose–response determination may be obtained by exposing the assay medium to loaded polymer for different times. With longer exposure time, more chemical will have desorbed into the assay medium, leading to a higher concentration. Time required for reaching equilibrium between the dosing phase and assay medium depends on physico-chemical properties of chemicals as well as the dimensions of the experimental set-up (Brown et al., 2001; Kramer et al., 2007; Kwon et al., 2007b; Jahnke et al., 2008).

Theoretical prediction of mass transfer is essential to enhance the applicability of this emerging method. In order to provide theoretical interpretation of the new dosing technique, we investigated how fast chemical substances migrate from PDMS to the assay medium. Five polycyclic aromatic hydrocarbons (PAHs) with various molecular size and hydrophobicity were chosen as model hydrophobic compounds. We used membrane vesicles of approximately 100 nm diameter as surrogates for any type of biomaterial in an in-vitro assay. Two types of membrane vesicles were used: liposomes composed of phosphatidylcholine, which are pure phospholipid bilayer vesicles, and isolated bacterial membrane vesicles called "chromatophores" composed of approximately 30% membrane lipids and 70% proteins (Escher et al., 1997).

Liposome–water and chromatophore–water partition coefficients were obtained using a PDMS depletion method (Ter Laak et al., 2005) and literature PDMS–water partition coefficients (K_{PDMSw}) (Kwon et al., 2007b). Apparent mass transfer resistances in the PDMS phase were directly measured and mass transfer resistance in the suspension was derived from these data and the measured overall resistance of desorption. Three simple kinetic models were evaluated based on the experimental data to explain time–concentration profile of test chemicals in the model assay media for a bioassay.

2. Theory

The two-compartment mass transfer model comprised of PDMS and the suspension of assay medium containing biomaterial is described as (Kwon et al., 2007b):

$$\frac{dC_{sus}}{dt} = k_d \frac{V_{PDMS}}{V_{sus}} C_{PDMS} - k_a \frac{V_{PDMS}}{V_{sus}} C_{sus} \quad (1)$$

$$\frac{dC_{PDMS}}{dt} = k_a C_{sus} - k_d C_{PDMS} \quad (2)$$

where C_{PDMS} (mol m_{PDMS}^{-3}) and C_{sus} (mol $m_{suspension}^{-3}$) are concentrations of a chemical in PDMS and the assay medium, V_{PDMS} and V_{sus} are the volumes of PDMS and the suspension (m^3), k_d is the desorption rate constant (s^{-1}) and k_a is the absorption rate constant (s^{-1} $m_{suspension}^{-3} m_{PDMS}^{-3}$). If the partition coefficient between PDMS and the assay medium, $K_{PDMSsus}$ ($m_{suspension}^{-3}$ m_{PDMS}^{-3}), has been independently measured, k_a should be equal to $K_{PDMSsus}$ k_d:

$$\frac{dC_{sus}}{dt} = k_d \frac{V_{PDMS}}{V_{sus}} (C_{PDMS} - K_{PDMSsus} C_{sus}) \quad (3)$$

$$\frac{dC_{PDMS}}{dt} = k_d (K_{PDMSsus} C_{sus} - C_{PDMS}) \quad (4)$$

If no chemical is initially present in the medium (i.e., $C_{sus}(t = 0) = 0$), the analytical solutions of Eqs. (3) and (4) are:

$$C_{sus} = \frac{C_{PDMS,0} \frac{V_{PDMC}}{V_{sus}}}{1 + K_{PDMSsus} \frac{V_{PDMS}}{V_{sus}}} \left[1 - \exp\left(-\left(K_{PDMSsus} \frac{V_{PDMS}}{V_{sus}} + 1\right)\right) k_d t\right] \quad (5)$$

$$C_{PDMS} = \frac{C_{PDMS,0}}{1 + K_{PDMSsus} \frac{V_{PDMS}}{V_{sus}}} \left[K_{PDMSsus} \frac{V_{PDMS}}{V_{sus}} + \exp\left(-\left(K_{PDMSsus} \frac{V_{PDMS}}{V_{sus}} + 1\right)\right) k_d t\right] \quad (6)$$

where $C_{PDMS,0}$ is the initial concentration in PDMS (mol m_{PDMS}^{-3}). Detailed derivation for Eqs. (5) and (6) are provided in our previous paper (Kwon et al., 2007b). The desorption rate constant, k_d, is the only parameter needed to be determined to predict time-dependent mass transfer because $K_{PDMSsus}$ can be easily determined in an independent experiment. The overall mass transfer resistance, $R_{overall}$ (m^{-1} s), can be defined as:

$$k_d = \frac{1}{R_{overall}} \frac{A}{V_{PDMS}} \quad (7)$$

where A is the interface area (m^2). Due to the additivity of mass transfer resistances in a simple film diffusion model, we may divide $R_{overall}$ into two terms, R_{PDMS} and R_{sus}, which are mass transfer resistances in PDMS and the assay medium, respectively. R_{PDMS}, can be experimentally determined by directly contacting two PDMS disks and measuring the rate of migration from one to the other (Mayer et al., 2005), while R_{sus} can be obtained by subtracting R_{PDMS} from $R_{overall}$ obtained using Eq. (7) with experimentally determined k_d.

$$R_{overall} = R_{sus} + R_{PDMS} \quad (8)$$

If we assume that diffusion occurs in a finite thickness of boundary layer, R_{PDMS} is

$$R_{PDMS} = \frac{\delta_{PDMS}}{D_{PDMS}} \quad (9)$$

where δ_{PDMS} is the thickness of diffusion boundary layer in PDMS (m) and D_{PDMS} is the diffusion coefficient in PDMS (m^2 s^{-1}). δ_{PDMS} can be regarded as one-half the thickness of a PDMS square if the concentration gradient is linear in PDMS (Ai, 1997; Salaün and Buffle, 2004).

R_{sus} can be characterized by two limiting cases. Since exchange of hydrophobic chemicals between water and small suspended particles ($d < 100$ nm) is thought to be instantaneous, one may assume that the solute–vesicle complexes are completely labile and the suspension behaves like a solvent mixture such as methanol/water mixture (model I). If the boundary layer can be characterized by mixed solvents, R_{sus} can be regarded as:

$$R_{sus} = \frac{\delta_{sus}}{D_{sus}} K_{PDMSsus} \quad (10)$$

where δ_{sus} is the boundary layer thickness of assay medium and D_{sus} is the effective diffusion coefficient of the solute in the particle suspension incorporating diffusivities of free and associated species to vesicles. Although the thickness of laminar boundary layer is known to be proportional to $D^{1/3}$ (Levich, 1962), it is assumed that δ_{sus} is the same for all selected PAHs because their diffusivities do not differ by a factor of 1.4.

Alternatively, one may assume that the aqueous boundary layer still limits overall mass transfer of hydrophobic chemicals even though the aqueous boundary layer is reduced as compared to pure water by adding bio-suspensions (model II). A solute molecule in PDMS must dissolve in water before it eventually is transferred into bio-suspensions in the bulk. Then, R_{sus} can be determined by the thickness of reduced aqueous boundary layer (δ), aqueous diffusion coefficient (D_w), and partition coefficient between PDMS and water (K_{PDMSw}):

$$R_{sus} = \frac{\delta}{D_w} K_{PDMSw} \quad (11)$$

Note that δ is a conceptual thickness not an actual aqueous boundary layer, therefore the subscript w was omitted to differentiate it

from δ_w in aqueous solution. Aqueous diffusion coefficients can be estimated by the molecular weight of a solute (M) according to Schwarzenbach et al. (2003) by:

$$D_w = \frac{2.7 \times 10^{-8}}{M^{0.71}} \quad (12)$$

Finally there is a third, combined model possible where the overall mass transfer resistance in the suspension is divided into two resistances, one related to aqueous diffusion and the other to diffusion in a solvent mixture. In this model, solute dissolved in PDMS should dissolve into a layer of unstirred water before entering the suspension that behaves like a completely mixed solvent. In other words, one may assume a series of diffusion resistances in pure water and in the completely mixed suspension where the physical boundary layer is determined by external agitation. This model can be mathematically described as a hybridized model of model I and II, as follows:

$$R_{sus} = f_1 \frac{\delta_w}{D_{sus}} K_{PDMSsus} + f_2 \frac{\delta_w}{D_w} K_{PDMSw} \quad (13)$$

where δ_w is the thickness of boundary layer, f_1 is the fraction of the resistance in the suspension side due to diffusion in a mixed solvent and f_2 is the fraction due to aqueous diffusion and equals to $1 - f_1$.

3. Experimental

3.1. Materials

Five polycyclic aromatic hydrocarbons (PAHs), phenanthrene, fluoranthene, benzo[*a*]pyrene, dibenzo[*a,c*]anthracene, benzo[*ghi*]-perylene, with three to six aromatic rings were chosen as model hydrophobic chemicals. They were of high purity (over 98%) and purchased from Fluka (Buchs, Switzerland) and Sigma–Aldrich (St. Louis, MO, USA). Medical grade poly(dimethylsiloxane) (PDMS) sheet (1 mm thick, density of 1170 kg m^{-3}; Specialty Silicone Products, Inc., Ballston, Spa, NY, USA) was cut into various size disks for partitioning experiments and into 10 × 10 mm squares for kinetic dosing purpose. This specific PDMS material has been used in several partitioning and diffusion studies (Kwon et al., 2007b; Mayer et al., 2007; Jahnke et al., 2008). PDMS squares and disks were prepared and stored before use as described earlier (Kwon et al., 2007b). Liposomes were made of palmitoyl-oleoyl-phosphatidylcholine (POPC) (Avanti Polar Lipids, Alabaster, AL, USA), in 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) at pH 7 and 100 mM KCl using a membrane filtration method (Kaiser and Escher, 2006). The resulting unilamellar vesicles had average diameters between 98 and 108 nm. Membrane vesicles ("chromatophores") were prepared and characterized as described by Escher et al. (1997). Chromatophores have a diameter of approximately 50 nm (Casadio et al., 1988). The phospholipid content was 32.3 ± 0.5% as determined by quantification of organic phosphorous (as total minus inorganic phosphorous, courtesy of the inorganic laboratory of Eawag). For measurement of partition coefficients, frozen chromatophores were thawed and diluted in the MOPS buffer described above. Suspended particle concentrations used in this study were 1.73–1.85 $mg_{dry\ weight}$ mL^{-1} for POPC liposomes and 1.62 ± 0.11 $mg_{dry\ weight}$ mL^{-1} for chromatophores.

3.2. Chemical analyses

All five selected PAHs were analyzed using an HPLC system equipped with a Dionex P680 pump and an ASI-100 autosampler (Dionex Softron GmbH, Germering, Germany). They were separated on a C18 Supelcosil LC-PAH column (150 mm × 4.6 mm, 5 μm, Supelco, Bellefonte, PA, USA) in a column oven maintained at 40 °C. They were measured using an RF-2000 fluorescence detector (Dionex) with the excitation wavelength (λ_{ex}) of 275 nm and the emission wavelength (λ_{em}) of 350 nm for phenanthrene, λ_{ex} = 260 nm and λ_{em} = 420 nm for fluoranthene, and λ_{ex} = 290 nm and λ_{em} = 430 nm for benzo[*a*]pyrene, dibenzo[*a,c*]anthracene, and benzo[*ghi*]perylene.

3.3. Determination of partition coefficients

Partition coefficients between POPC liposomes and buffer and between chromatophores and buffer were determined using the PDMS depletion method (Ter Laak et al., 2005). Shortly, PDMS disks were cut into various sizes (total volume of 1.2,5.2,28.7 μL), cleaned and preloaded with PAHs using methanol/water (60/40) loading solution containing PAHs mixture as previously described (Kwon et al., 2007b). The initial concentration in PDMS ranged from 20 to 400 μmol L^{-1}. After apparent equilibrium was attained (three days), PDMS disks were removed and extracted by shaking with acetonitrile at 150 rpm for 2 h. Acetonitrile solution was injected into the HPLC system described above. Assuming 100% mass balance, the equilibrium concentration in PDMS (C_{PDMS}) over the initial concentration in PDMS ($C_{PDMS,0}$) is given by

$$\frac{C_{PDMS}}{C_{PDMS,0}} = \frac{1}{1 + \frac{DF}{K_{PDMSsus}}} \quad (14)$$

where DF is the dilution factor (volume suspension to volume PDMS) and $K_{PDMSsus}$ is a partition coefficient between PDMS and chromatophore suspension. $K_{PDMSsus}$ values were obtained by a non-linear regression using Eq. (14) from at least six different dilutions with at least four replicates. Assuming that activity coefficients of PAHs in the buffer solution are similar to those in deionized water, partition coefficient between liposomes (or chromatophores) and buffer (K_{lipw} or K_{cphw}) were estimated using K_{PDMSw} by

$$K_{lipw} = \frac{\frac{K_{PDMSw}}{K_{PDMSsus}} - 1}{C_{lip}} \quad (15)$$

where K_{lipw} (or K_{cphw}) is expressed in units of L_{water}/kg_{lipid} and C_{lip} is in kg_{lipid}/L_{water}. Values of PDMS-water partition coefficients (K_{PDMSw}) were taken from Kwon et al. (2007b) obtained by the dynamic permeation method.

3.4. Determination of desorption rate constants and mass transfer resistances

In order to model delivery rates from PDMS to bio-suspensions, the mass transfer resistances were measured in both phases. A "donor" PDMS disk loaded with chemicals was in direct contact with a clean "acceptor" PDMS disk in a closed glass vessel (Fig. S1, Supplementary material). After pre-determined times from 0.5 to 27 h, the donor and the acceptor disks were separated and extracted using acetonitrile to measure chemical concentration in PDMS. The volume of acetonitrile was at least 35 times the volume of PDMS disks and over 98% of extractable PAHs were extracted from the first extraction. The mass transfer resistance in PDMS was obtained by Eq. (7).

Fig. 1 shows the experimental set-up used for evaluating dosing kinetics. One PDMS square loaded with a mixture of 5 PAHs was placed at the bottom of a quartz cuvette containing 2 mL of assay medium (i.e., chromatophore suspension or POPC suspension as surrogate biological phases). In order to enhance the desorption rate, suspension was stirred at 200 rpm using a tumble stirrer (VP710, V&P Scientific, Inc., San Diego, CA, USA) with a stirring disk (d = 7.95 mm, 0.635 mm thick) as shown in Fig. 1. To obtain C_{sus}, both the depletion of concentration in PDMS and extractable

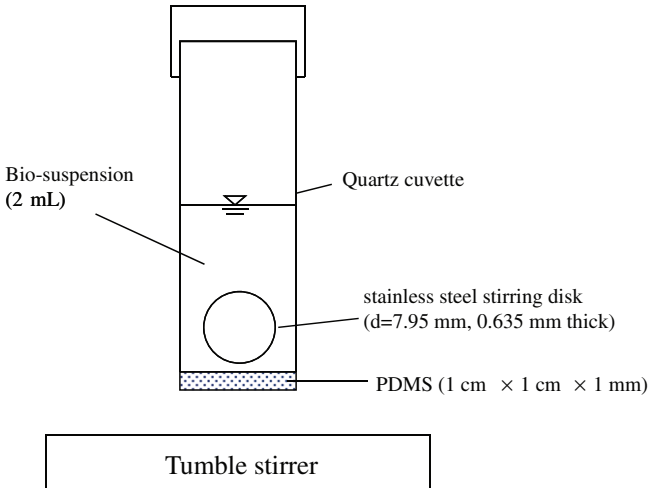

Fig. 1. Schematic diagram of the partition controlled delivery system used in this study.

concentration in the suspension were measured. PDMS squares were extracted as described earlier using acetonitrile. The chromatophore suspension was denatured by adding 4-fold ice-cold acetonitrile and the denatured suspension was centrifuged and precipitated down at 4000*g* for 10 min. The supernatant was taken for chemical analysis to quantify chemical concentration in the suspension. It was not possible to precipitate the liposome suspension because liposomes do not contain any proteins. The total mass balance calculated from measured C_{PDMS} and C_{sus} was typically around 100% for the first 20 h of the desorption experiment and then decreased to 70–85% with extended exposure time (see Fig. S2, Supplementary material for details). Given that mass balance was close to 100% for the chromatophores, we also assumed 100% for liposomes for the liposome–water partitioning experiments and k_d was fitted with equation Eq. (6), after plotting $C_{PDMS}/C_{PDMS,0}$ as a function of time. The desorption rate constant, k_d, was obtained using Eqs. (5) and (6) by the non-linear regression using GraphPad Prism software (Graphpad Software, Inc., La Jolla, CA, USA) and standard errors of resistances were obtained by error propagation.

4. Results and discussion

4.1. Equilibrium partition coefficients

Fig. 2A and B shows the determination of partition coefficients between PDMS and POPC liposomes and between PDMS and chromatophore suspension, respectively. Table 1 summarizes experimentally determined $K_{PDMSsus}$ and calculated partition coefficients between suspended vesicles and water for POPC liposomes and chromatophore suspensions with literature partition coefficients, log K_{ow} and log K_{PDMSw}. Values of log K_{lipw} obtained in this study were very close to those reported by Jonker and van der Heijden using PDMS-SPME fibers (Jonker and van der Heijden, 2007). They reported 5.07, 5.67, and 8.04 for phenanthrene, fluoranthene, and benzo[*ghi*]perylene, respectively.

Partition coefficients between chromatophores and water (K_{cphw}) were lower than K_{lipw} approximately by one order of magnitude. Because partitioning of hydrophobic chemicals is dominated by lipid fraction (Schwarzenbach et al., 2003) and chromatophores used in this study contain 31.7% phospholipids, lipid normalized log K_{cphw} values were 0.50 log unit higher than values obtained based on dry weight of chromatophores as shown in Table 1.

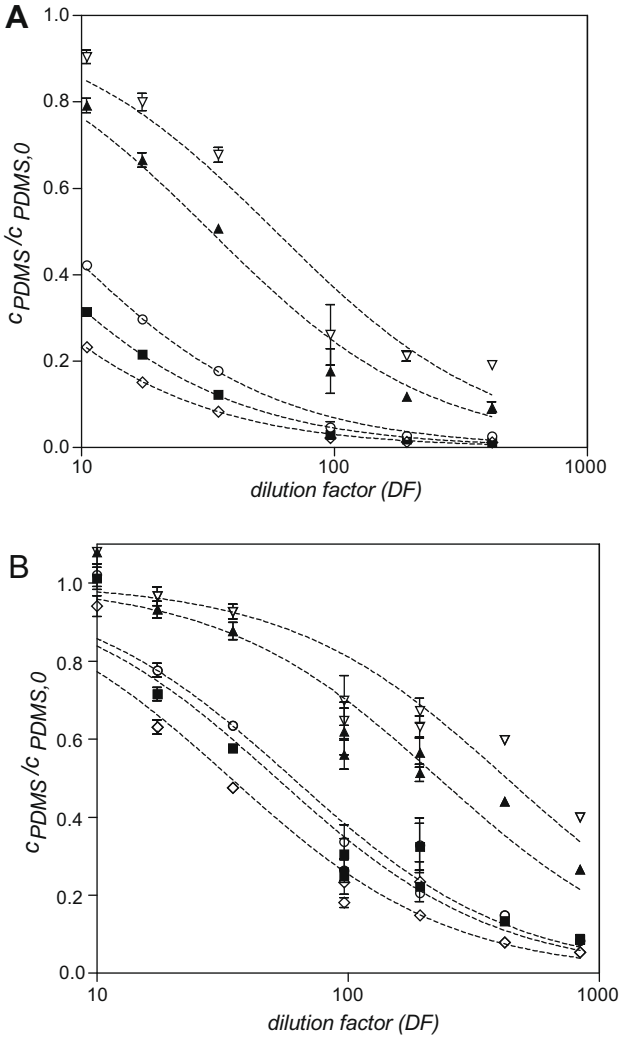

Fig. 2. Determination of partition coefficients (A) between PDMS and liposome suspension (1.73 mg L^{-1}) and (B) between PDMS and chromatophores (1.62 mg L^{-1}) of (▽) phenanthrene, (▲) fluoranthene, (○) benzo[*a*]pyrene, (■) dibenzo[*a,c*]anthracene, (◇) benzo[*ghi*]perylene. Error bars denote standard errors and dashed lines represent non-linear fit using Eq. (14).

However, lipid-normalized chromatophore–water partition coefficients are still lower than K_{lipw} by a factor of 1.5 (phenanthrene) to 3.2 (dibenzo[*a,c*]anthracene), although the two partition coefficients are linearly correlated with r^2 greater than 0.99 (Fig. S3, Supplementary material). Partitioning of hydrophobic chemicals into proteins like bovine serum albumin is typically by a factor of 20 smaller than partitioning into octanol (de Bruyn and Gobas, 2007). Consequently, although chromatophores contain almost 70% proteins, their contribution to partitioning would be negligible and it cannot explain the experimental differences because protein binding would increase lipid-normalized K_{cphw}. Thus, experimental differences may be explained by differences in the fluidity of lipid membranes. Chromatophore membranes contain both saturated and unsaturated phospholipids and membrane proteins (Birrell et al., 1978). They are not as fluidic as POPC (16:0,18:1) liposomes, which is one of the most fluidic phospholipids with the main transition temperature of 4 °C (Jain, 1988). Table 1 also shows that the difference between K_{lipw} and K_{cphw} increases with increasing ring numbers or molecular weight, suggesting that effects of solute size on partitioning is larger for less fluidic membranes. This is consistent with recent experimental findings that enthalpy required for cavity creation is significantly larger for highly-organized dipalm-

Table 1
Partition coefficients of the selected PAHs.

Chemical	log K_{ow}[a]	log K_{PDMSw}[b]	POPC suspension			Chromatophore suspension			
			$K_{PDMSsus}$	r^2	log K_{lipw} (L kg_{lip}^{-1})	$K_{PDMSsus}$	r^2	log K_{cphw} (L $kg_{dry\ weight}^{-1}$)	log K_{cphw} (L kg_{lip}^{-1})
Phenanthrene	4.52	3.87	58.7 ± 5.7	0.92	4.86	428 ± 37	0.73	4.00	4.49
Fluoranthene	5.20	4.32	32.5 ± 2.1	0.96	5.57	230 ± 17	0.84	4.74	5.24
Benzo[*a*]pyrene	6.35	5.09	7.42 ± 0.19	0.98	6.98	60 ± 4.4	0.88	6.10	6.59
Dibenzo[*a,c*]anthracene	6.17	5.41	4.76 ± 0.11	0.99	7.49	52 ± 4	0.88	6.48	6.98
Benzo[*ghi*]perylene	6.90	5.36	3.15 ± 0.05	0.99	7.62	34 ± 2	0.90	6.62	7.11

[a] Values of log Kow are recommended values in LOGKOW database (Sangster Research Laboratory, 2008).
[b] Values obtained by ABL permeation method (Kwon et al., 2007b).

itoyl-phosphatidylcholine (DPPC) than fluidic POPC (Kwon et al., 2007a).

4.2. Mass transfer resistances

Mass transfer resistances in PDMS (R_{PDMS}) were obtained from the direct contact experiment of the "donor" PDMS with the "acceptor" PDMS. The overall mass transfer resistance, R_{PDMS} was calculated using Eq. (7) from k_d obtained by a non-linear regression using Eqs. (5) and (6) as shown in Fig. 3. Then, the mass transfer resistances in the suspension (R_{sus}) were calculated by subtracting R_{PDMS} from $R_{overall}$ obtained in the presence of suspension. Table 2 summarizes all values. Mayer et al. (2005) used a similar system to measure the rate of diffusion in PDMS using fluoranthene as a model compound. The first-order desorption rate constant was 2.24 h^{-1} with corresponding mass transfer resistance of 2.68×10^6 m^{-1} s. Because they used thinner PDMS (600 μm), the resistance should be 60% of this study. The measured resistance for fluoranthene (4.14×10^6 m^{-1} s) is very close to theirs (Mayer et al., 2005). Diffusion coefficients in PDMS estimated using Eq. (9) are 1.7×10^{-10}, 1.2×10^{-10}, 0.92×10^{-10}, 0.56×10^{-10}, and 0.74×10^{-10} m^2 s^{-1} for phenanthrene, fluoranthene, benzo[*a*]pyrene, dibenzo[*a,c*]anthracene, benzo[*ghi*]perylene, respectively. They are in good agreement with measured diffusion coefficients in various commercial PDMS (Rusina et al., 2007).

R_{sus} values for POPC liposome suspensions are very similar for all compounds, with an average of $3.71(\pm1.43) \times 10^7$ m^{-1} s. In contrast the R_{sus} values for chromatophore suspensions increase from 1.8×10^7 m^{-1} s to 23×10^7 m^{-1} s generally with increasing hydrophobicity. This observation is consistent with previous work by Mayer et al. (2007), where the velocity rate constants of PAHs in water showed a large dependence on the hydrophobicity of the PAHs, while in presence of surfactants or hydroxypropyl-ß-cyclodextrin, almost no hydrophobicity dependence could be observed. In addition, the main mass transfer resistance remained on the solution side for both bio-suspensions. This makes PDMS a good dosing material particularly for tests that require an increased mass transfer into a solution with high content of biomaterials.

According to model I, dividing R_{sus} by $K_{PDMSsus}$ gives δ_{sus}/D_{sus} (Eq. (10)). This term (δ_{sus}/D_{sus}) increases with increasing hydrophobicity by two orders of magnitude and the changes are dominated by the changes in hydrophobicity, not by the measured resistances. δ_{sus}/D_{sus} changes from $10^{3.73}$ m^{-1} s (phenanthrene) to $10^{7.14}$ m^{-1} s (benzo[*ghi*]perylene) for POPC suspension and from $10^{4.62}$ m^{-1} s (phenanthrene) to $10^{6.85}$ m^{-1} s (benzo[*ghi*]perylene) for chromatophores. Because δ_{sus} is determined by the external agitation strength of the medium, not by the chemical property of solutes, and the effective diffusion coefficients (D_{sus}) in the well-mixed solvent should be size-dependent but not vary by orders of magnitude, δ_{sus}/D_{sus} should not vary as much as experimentally determined. In addition, δ_{sus}/D_{sus} values are 1–12 times larger in liposomes than in chromatophores for a given compound. Concentrations of membranes used per gram dry weight were very close to each other and the average diameter of vesicles is approximately 100 nm for liposomes and 50 nm for chromatophores (Casadio

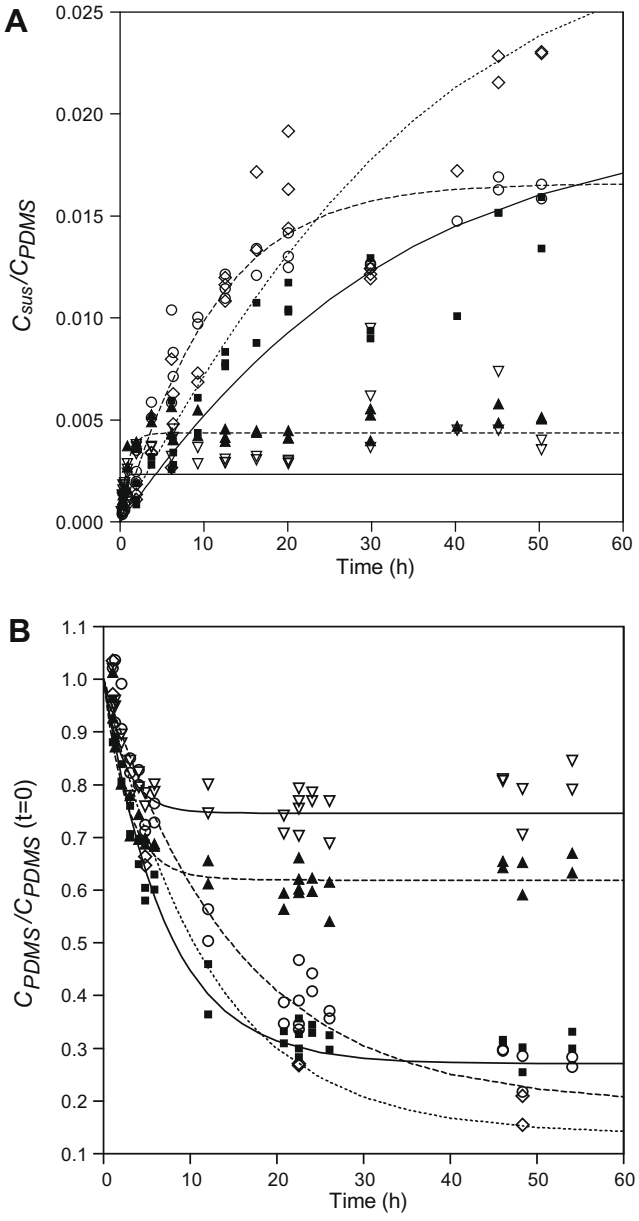

Fig. 3. Determination of the desorption rate constant (k_d) of (▽) phenanthrene, (▲) fluoranthene, (○) benzo[*a*]pyrene, (■) dibenzo[*a,c*]athracene, (◇) benzo[*ghi*]perylene from PDMS into (A) 1.62 mg mL^{-1} chromatophore suspension and (B) into 1.73 and 1.85 mg mL^{-1} POPC suspension. Experimental $K_{PDMSsus}$ values determined in the previous step were used.

Table 2
The desorption rate constants (k_d), corresponding mass transfer resistances in PDMS (R_{PDMS}), overall mass transfer resistances ($R_{overall}$) and calculated mass transfer resistances in the assay medium (R_{med}) for POPC liposomes and chromatophores.

Chemical	Direct contact experiments		POPC suspension			Chromatophore suspension		
	k_d (h^{-1})	R_{PDMS} ($\times 10^7$ m^{-1} s)	k_d (h^{-1})	$R_{overall}$ ($\times 10^7$ m^{-1} s)	R_{sus} ($\times 10^7$ m^{-1} s)	k_d (h^{-1})	$R_{overall}$ ($\times 10^7$ m^{-1} s)	R_{sus} ($\times 10^7$ m^{-1} s)
Phenanthrene	0.615 ± 0.028	0.293 ± 0.013	0.104 ± 0.016	3.47 ± 0.53	3.18 ± 0.53	0.173 ± 0.101	2.08 ± 1.21	1.78 ± 1.21
Fluoranthene	0.435 ± 0.024	0.414 ± 0.023	0.136 ± 0.012	2.64 ± 0.23	2.23 ± 0.23	0.096 ± 0.013	3.74 ± 0.50	3.33 ± 0.50
Benzo[*a*]pyrene	0.333 ± 0.019	0.541 ± 0.031	0.053 ± 0.002	6.77 ± 0.76	6.23 ± 0.76	0.027 ± 0.001	13.4 ± 7.0	12.9 ± 7.0
Dibenzo[*a*,*c*]anthracene	0.202 ± 0.012	0.893 ± 0.055	0.103 ± 0.006	3.48 ± 0.67	2.59 ± 0.09	0.012 ± 0.001	31.2 ± 2.0	30.4 ± 2.0
Benzo[*ghi*]perylene	0.267 ± 0.017	0.674 ± 0044	0.072 ± 0.003	5.00 ± 0.21	4.33 ± 0.21	0.015 ± 0.001	23.6 ± 1.7	22.9 ± 1.7

Table 3
Model parameters of the two limiting cases that describe the processes at the polymer–suspension interface for the five PAHs.

Chemical	POPC suspension			Chromatophores		
	Model I log (δ_{sus}/D_{sus})	Model II δ_w (μm)	Model III f_1 f_2	Model I log (δ_{sus}/D_{sus})	Model II δ_w (μm)	Model III f_1 f_2
Phenanthrene	5.73	2.92	0.806 0.194	4.62	1.64	0.891 0.109
Fluoranthene	5.84	0.66	0.956 0.044	5.16	0.99	0.934 0.066
Benzo[*a*]pyrene	6.60	0.13	0.982 0.018	6.33	0.56	0.963 0.037
Dibenzo[*a*,*c*]anthracene	7.09	0.11	0.997 0.003	6.78	0.59	0.961 0.039
Benzo[*ghi*]perylene	7.14	0.09	0.994 0.006	6.83	0.50	0.967 0.033

et al., 1988). This difference should be at most a factor of 2 since the density of both biomaterials is similar. This comparison indicates that it is not reasonable to assume that bio-suspensions are completely mixed with water in a conceptual film at the PDMS–suspension interface. It may be possible to estimate D_{sus} assuming that labile PAH-vesicle complexes using vesicle size and partition coefficients (for an example calculation, see Part A, Supplementary material). However, these calculations were not used for the evaluation of the model because they require too many assumptions and dataset was too limited to evaluate such a theoretical model.

Using model II, the thickness of the aqueous boundary layer (δ) can be calculated by Eq. (11) as shown in Table 3. Although the δ values decreases with increasing hydrophobicity, they differ not as significantly as δ_{sus}/D_{sus} values from model I. The variation of δ is within a factor of 33 and 3.3 for POPC liposomes and chromatophores. Modeled δ generally decreased with increasing partition coefficient, K_{lipw} or K_{cphw}. The decrease of δ with increasing hydrophobicity has been observed for kinetics of passive sampling of PCBs and brominated flame retardants into PDMS passive sampler (Ter Laak et al., 2008).

The decrease of δ with increasing hydrophobicity may be rationalized by that the average diffusion path is longer for less hydrophobic chemicals because they have lower possibility of sequestration by the biomembranes than more hydrophobic chemicals.

A hybrid model (model III) may be used, where solutes dissolved in PDMS first dissolve into and diffuse through water before they distribute between free and bound forms. As shown in Eq. (13), it is required to estimate both boundary layer thickness (δ_w) and effective diffusion coefficients in the suspension as a completely mixed solvent (D_{sus}) in order to obtain f_1 and f_2 from the experimental resistances. Physical thickness of the aqueous boundary layer should be around 10–20 μm based on our previous experiment using de-ionized water in a similar experimental setup (Kwon et al., 2007b) and the D_{sus} values can be assumed smaller than D_w because of higher viscosity. Thus, we set δ_w to 15 μm and D_{sus} to 1/5 D_w. These assumptions are conceptual because the diffusion coefficients should not only decrease due to increase in viscosity but also due to change of the diffusing species. Diffusion coefficients of free PAHs, which might dominate near PDMS surface, are much higher compared to bound forms that might dominate further away from PDMS surface. Nevertheless the conceptual model serves its purpose to evaluate the contribution of the two limiting cases.

Based on these assumptions, calculated f_1 and f_2 values are as shown in Table 3. Considering the resulting contributions from model I and II, one can conclude that the overall mass transfer is dominated by aqueous diffusion and the contribution of aqueous diffusion term generally decreases with increasing hydrophobicity. Thus, the uncertainties originating from the assumption of D_{sus} did not affect much the overall estimate of aqueous diffusion. This analysis is also consistent with model II because δ in model II is not the physical thickness, but the conceptual thickness. The hydrophobicity effects are more dramatic for POPC suspension than for chromatophores as R_{sus} values changed significantly for chromatophores whereas they did not for POPC. Importance of the aqueous diffusion and effects of types of bio-suspension may be explained by non-uniform distribution of nano-sized suspensions near the surface as shown by Kanda et al. (2007).

4.3. Potential applications of passive dosing methods

The dynamic delivery method evaluated in this study is promising to predict aqueous concentration in any bioassay with bio-suspensions of proteins, membrane vesicles, or cells. In addition, this technique may improve current biodegradation tests of hydrophobic chemicals using enzyme mixture because of the high protein density in such assays. Finally, the concepts developed in this study may help rationalize the association of PAHs with engineered nanoparticles and how nanoparticles may serve for reducing bioavailability but also as transporters of pollutants to cells.

Time to reach equilibrium between dosing device and bio-suspensions depends on the hydrophobicity of the investigated PAHs and the type of bio-suspensions. Thus, the dosing technique appears to be suitable for less hydrophobic compounds (e.g., log K_{ow} up to 5) in the equilibrium mode, while for more hydrophobic compounds, one can exploit the kinetics of uptake into the biomaterial to derive kinetic dose–response curves, under the condition that the biological response is instantaneous or much faster than the dosing kinetics.

Examples for such applications are the Kinspec method to determine uncoupling and baseline toxicity (Escher et al., 1997) or pulse amplitude modulated fluorometry to determine the photosynthesis yield in green algae and plants (Schreiber et al.,

2007). A dose–response relationship can be obtained in a single well of a well-plate or another experimental device if one knows desorption kinetics as a function of exposure time and the corresponding biological response of the in vitro assay does not change significantly within the experimental time frame.

Linking this system to cell-based in-vitro assays is also feasible because cell lines typically grow in a protein-rich medium. For compounds of log K_{ow} > 5, it is possible to incubate the passive dosing device with the protein-rich medium up to a point where an equilibrium between bio-suspension and passive dosing device is reached. Then one may add cells and measure the toxicity. The sorptive capacity of the serum proteins is rather high (de Bruyn and Gobas, 2007), so that the addition of cells will not shift the equilibrium very much. Differences in the dosing kinetics into liposomes and chromatophores, despite the similar size of the colloidal particles, suggest that for each bioassay application the dosing kinetics need to be established prior to use.

Even if a biological response is not instantaneous such as in the ecotoxicity testing with whole animals, e.g., invertebrates like *Daphnia magna*, and lethality or reproduction endpoints, the proposed dosing system can be applied because the PDMS represents a large reservoir and different types of exposure scenarios can be simulated with the proposed system.

The dynamic passive dosing system proposed here is a very well defined system. In contrast, the common method applied to dose hydrophobic chemicals into bioassays, which is spiking of the chemical in a co-solvent, will result in an initially highly dynamic and undefined system. It might even be possible to overdose the system, so that freely dissolved concentrations temporarily exceed the solubility limit, which then would lead to micro-crystal formation. Dosing of hydrophobic chemicals will always be dynamic because it is the nature of very hydrophobic molecules that they exhibit slow kinetics. The proposed dosing system is superior to solvent spiking because it explicitly accounts for the dynamics of dosing. In conclusion, the proposed and characterized system offers a wide range of applications for dosing of hydrophobic chemicals into in-vitro bioassays.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2009.02.023.

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