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Summary

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Title:
Relationship between *in vivo* and *in vitro* toxicity of six types of carbonaceous nanoparticles

At present it is commonly hypothesised that the toxicity of ultrafine soot particles is largely driven by adsorbed redox-active components (e.g. polyaromatic hydrocarbons (PAHs)), which participate in redox-cycling reactions generating reactive oxygen species (ROS). These ROS can cause oxidative stress responses that may result in pulmonary or even systemic inflammation. Ultimately, these processes may promote the progression of atherosclerosis and precipitate acute cardiovascular responses ranging from increased blood pressure to myocardial infarction. The objective of the present study is to assess whether the inflammatory response of mice (*in vivo* toxicity) to combustion-derived nanoparticles (CDNPs) can be predicted by the combined information from a cell-free *in vitro* test for oxidative potency and an *in vivo* gene expression analysis targeting inflammation, stress and detoxification related genes.

Six types of carbonaceous particles were either purchased (Diesel SRM-1650a (DEP), PrintexG and Printex90) or generated in our laboratory. The latter comprised ultrafine carbon particles (UfCP) generated by spark-discharge, as well as soot particles with high and low organic content (SootH, SootL) produced by a well-controlled propane diffusion flame (CAST burner). These particle types had

widely varying (primary) particle diameter (10-50nm), organic content (OC; 1-20%) and specific BET surface area (43-800m^2/g). The *in vivo* toxicity was based on the response of BALB/cJ mice (21.1±1g) to particle exposure was based on the influx of polymorphonuclear neutrophils (PMNs) into the lungs 24h after intratracheal instillation of these particles (Stoeger et al., 2006). Using this data we defined the inflammatory efficacy (I_{Ef} [%PMN/µg]) as the 20% PMN effect level divided by the particle mass causing this effect level. The particles' innate oxidative potency (OxPot) was determined by a cell-free *in vitro* assay the consumption (in nmol) of an antioxidant standard (ascorbate).

We found that OxPot showed a strong linear correlation (R^2=0.77) with the *in vivo* inflammatory response (I_{Ef}) (Figure 1). The most obvious outlier was high-organics flame soot (SootH, OC=19%), for which the *in vitro* test clearly underestimated the *in vivo* toxicity. Since this was not observed at the same level for DEP, the other high-OC sample (OC=20%), OC alone could not account for this discrepancy. Gene expression analysis of 11 selected detoxification enzymes revealed that the only gene, which was specifically upregulated by SootH (3.9 fold) and DEP (1.6 fold), was the xenobiotic-metabolizing enzyme Cyp1a1. Cyp1a1 is well known to be highly inducible by bioavailable organic compounds, like aromatic hydrocarbons which. Thus the induction of Cyp1a1 by SootH, and to a lesser extent by DEP, indicated that the bioavailability of OC plays an important role for their toxicity. If we include the Cyp1a1 gene expression as independent parameter into a linear model, 94% of the observed variability in I_{Ef} can be explained by OxPot and Cyp1a1, while OxPot alone only accounts for 77% of the variability.

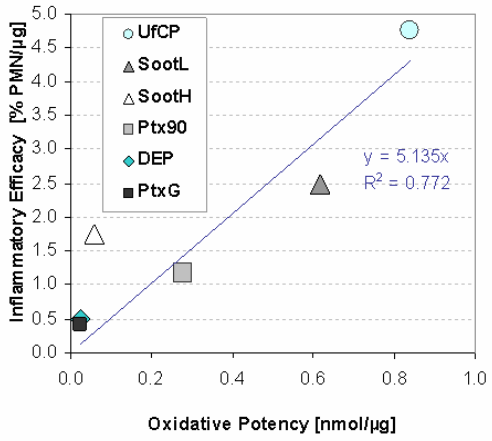

Figure 1. *In vitro* oxidative potency and *in vivo* inflammatory efficacy of the six types of CDNPs

Thus our data suggests that while organic coating might mitigate *in vivo* inflammatory response by possibly shielding the oxidative potency of the carbon core of CDNPs, CYP1A1 enzyme mediated biotransformation of organics may generate oxidative stress and thus enhance the *in vivo* inflammatory response.

Moreover the analysis presented here can be used to derive a simple, quantitative model for predicting the *in vivo* toxicity of CDNPs based on a simple *in vitro* assay for oxidative potency and (*in vivo*) *Cyp1a1* gene or protein expression. As a first approach we perform a linear regression (forced through

the origin) with the ascorbate based *in vitro* test (Ox_{Pot}) as independent and inflammatory efficacy as dependent parameter to obtain $I_{Ef} = 5.14 Ox_{Pot} [nmol/\mu g]$ (equation 1). As a second approach we model the *in vivo* inflammatory efficacy as linear function of both Ox_{Pot} and *Cyp1a1* gene expression (GE_{Cyp1a1} expressed as fold induction after instillation of 20µg particles) yielding, $I_{Ef} = 5.05 Ox_{Pot} [nmol/\mu g] + 0.509 (GE_{Cyp1a1} - 1)$ (equation 2), where we set GE_{Cyp1a1} to unity (no contribution from pathway 2), if *Cyp1a1* was down-regulated (<1). As seen from Figure 2 (left panel) the 1 parameter model (pathway 1 only; equ. 1) explains only 77% of the observed variability in inflammatory efficacy, while the 2 parameter model, which considers contributions from pathways 1 and 2 (equ. 2), explains 94%. This indicates that both pathways contribute to the observed inflammatory efficacy. Since the small data set (six data points) required limiting the number of independent fit parameters, we reduced the number of fit parameters to two by assuming linearity (through the origin) and independence of pathways 1 and 2 (see equ. 2).

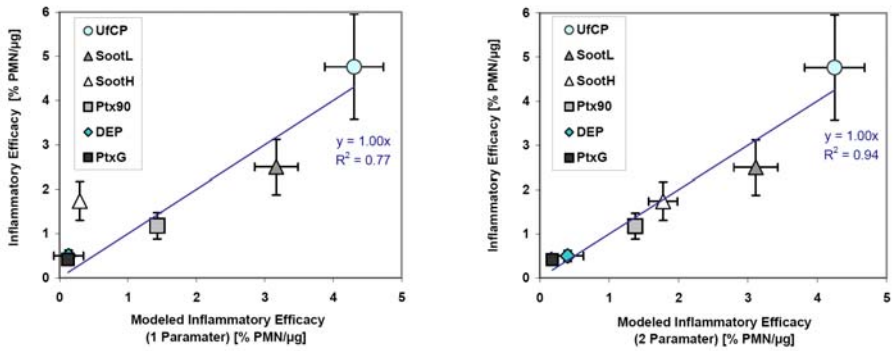

Figure 2: Predictive capacity of two simple linear models for the measured inflammatory efficacy.

While this is likely to be only a crude approximation for complex biological systems, the agreement between measured and modeled inflammatory efficacy is remarkable. Since our expression data is still derived from *in vivo* experiments, this model for toxicity prediction still requires animal exposures, although at a reduced amount. However, if future research will provide a means of obtaining *Cyp1a1* expression data from cell lines exposure, the toxicologcial model presented here may provide a true alternative to animal exposures.

Reference:

Stoeger et al. (2006). Environ. Health Presp., 114, 328-333

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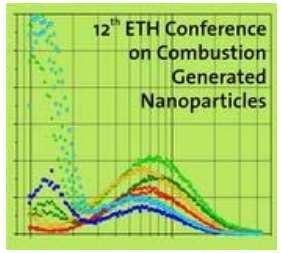
Institute of Inhalation Biology

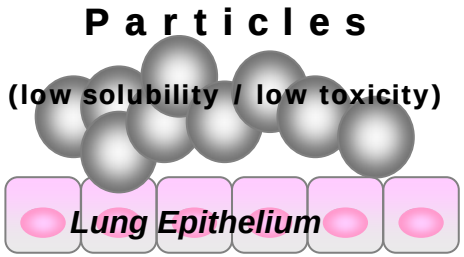
Zürich, 24/06/2008



HELMHOLTZ ASSOCIATION

Sources of Particle Induced Oxidative Stress

General Model



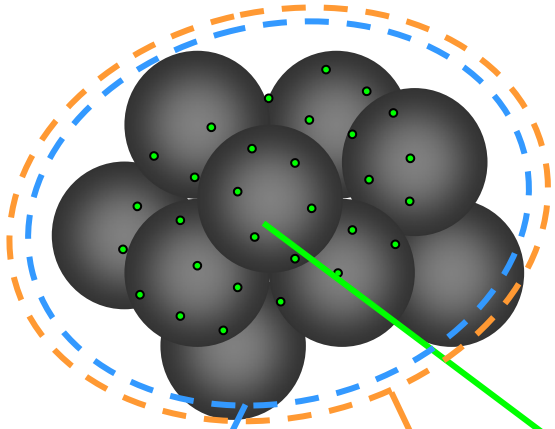
PM-Cell Interactions

Oxidative Stress / ROS

Inflammatory Response

Lung Disease
(fibrosis, lung cancer…)

…Cardiovascular Disease



Sources for Oxidative Stress

structural surface properties

organic compounds (PAH)

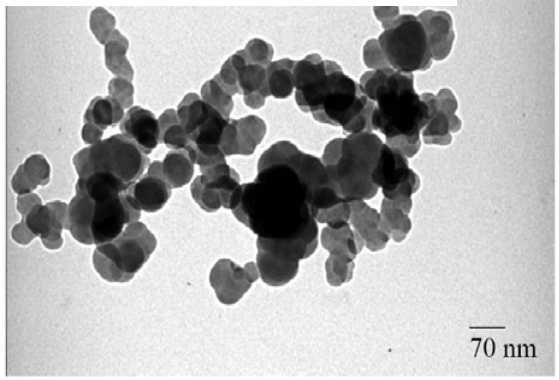
transition metals

Formation of Reactive Oxygen Species

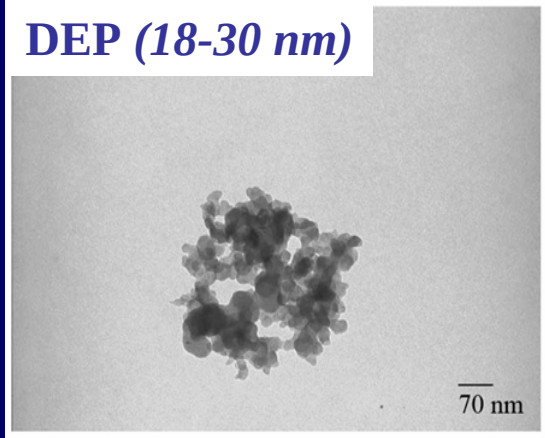
? Can we differentiate sources of oxidative stress / inflammation?

Investigated Carbonaceous Nanoparticles

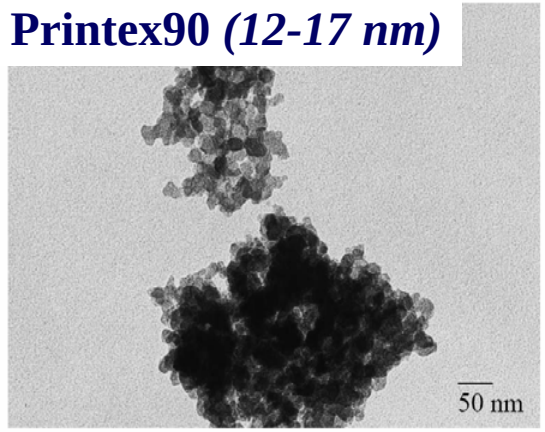
PrintexG ***(30-60 nm)***



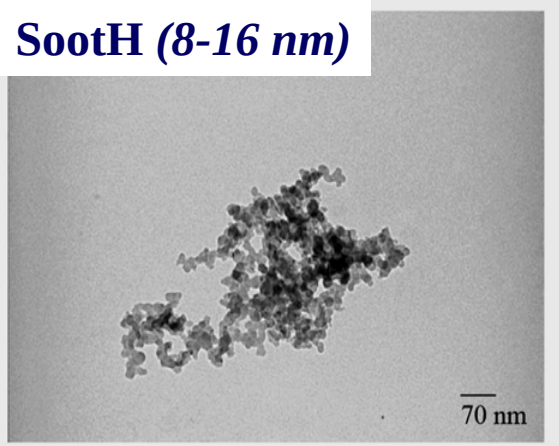
DEP ***(18-30 nm)***



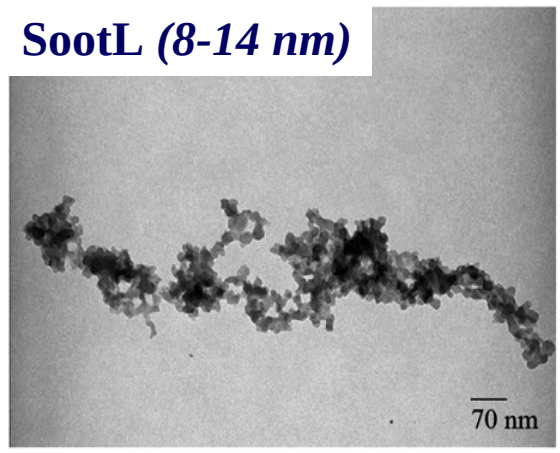
Printex90 ***(12-17 nm)***



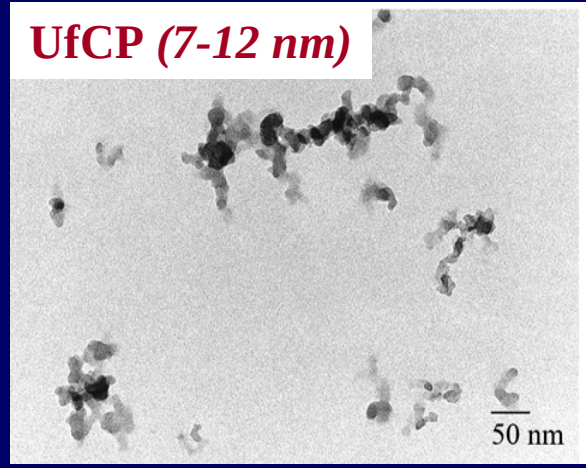
SootH ***(8-16 nm)***



SootL ***(8-14 nm)***



UfCP ***(7-12 nm)***

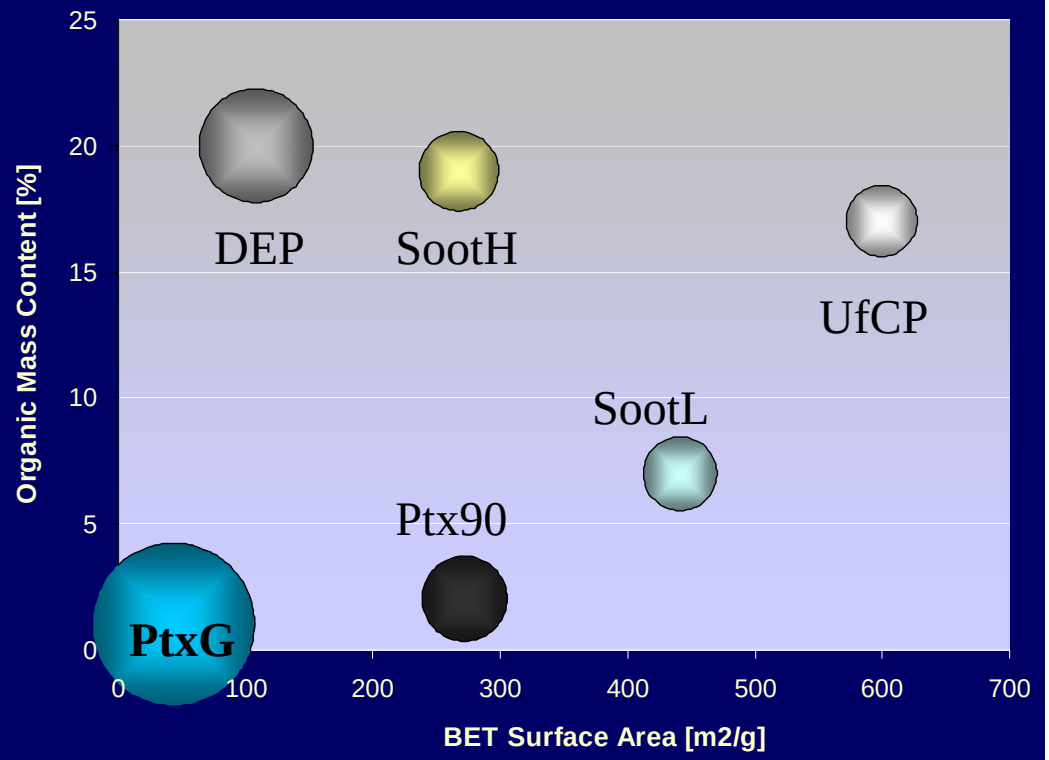


Investigated Carbonaceous Nanoparticles

Pigment Black	Spark Discharge	Flame Soot	Diesel Exhaust Particles
• Printex90 • PrintexG	• UfCP	• SootH • SootL	• DEP **SRM1650a**

Particle Characteristics

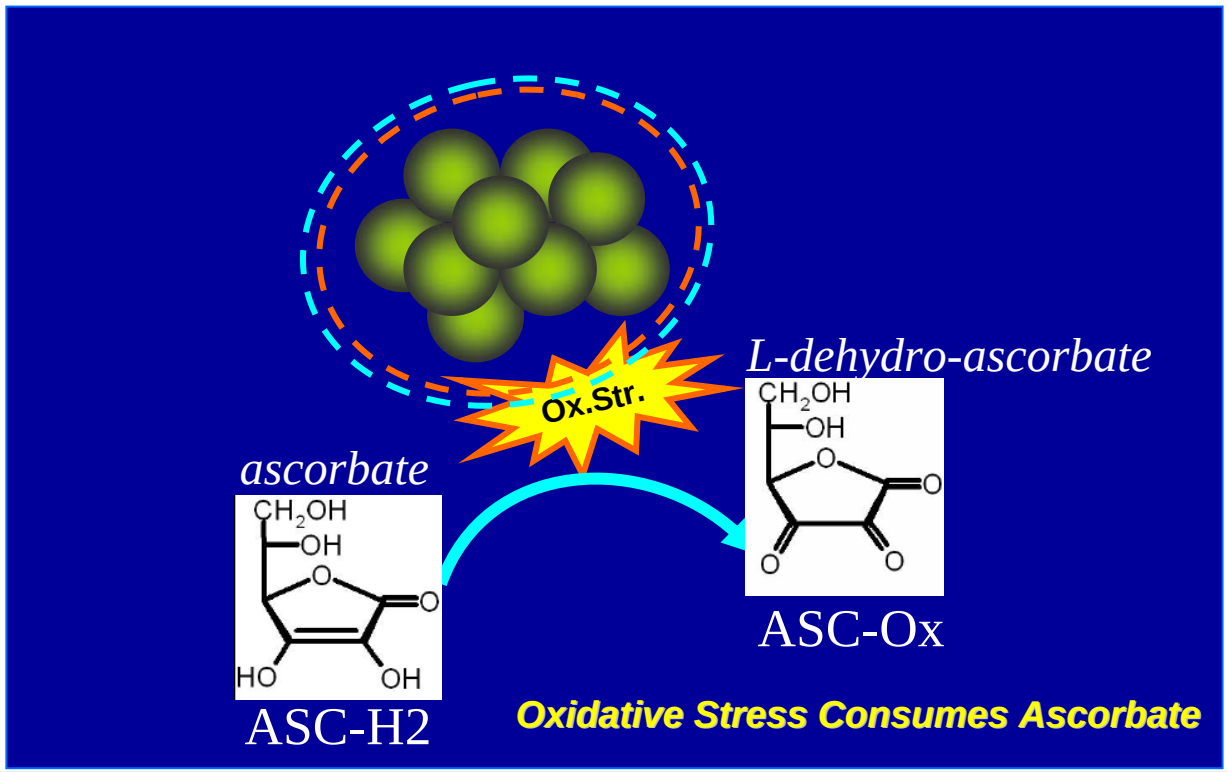
NPs	Diameter [nm]	Org. Content [%]	BET surface [m^2/g]
DEP	25	20	108
PtxG	51	1	43
Ptx90	14	2	272
SootH	12	19	268
SootL	11	7	441
UfCP	10	<4	600



How to Assess Oxidative Reactivity of Nanoparticles?

Oxidative potency of NPs assessed in a <u>cell free system</u>:

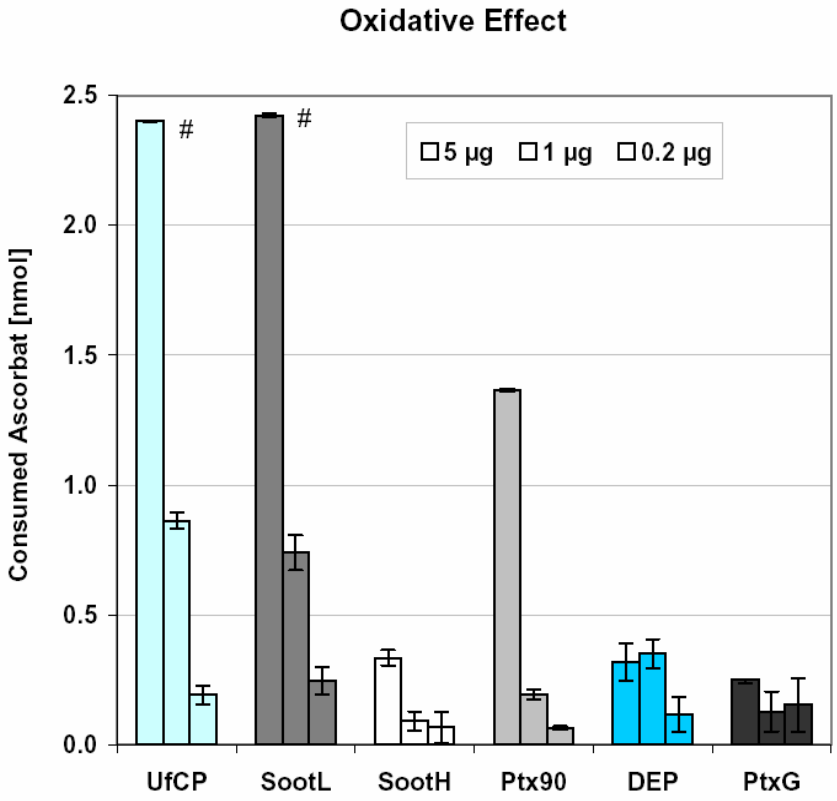
Consumption of the anti-oxidative capacity of *ascorbate* as a measure for the oxidative surface reactivity.

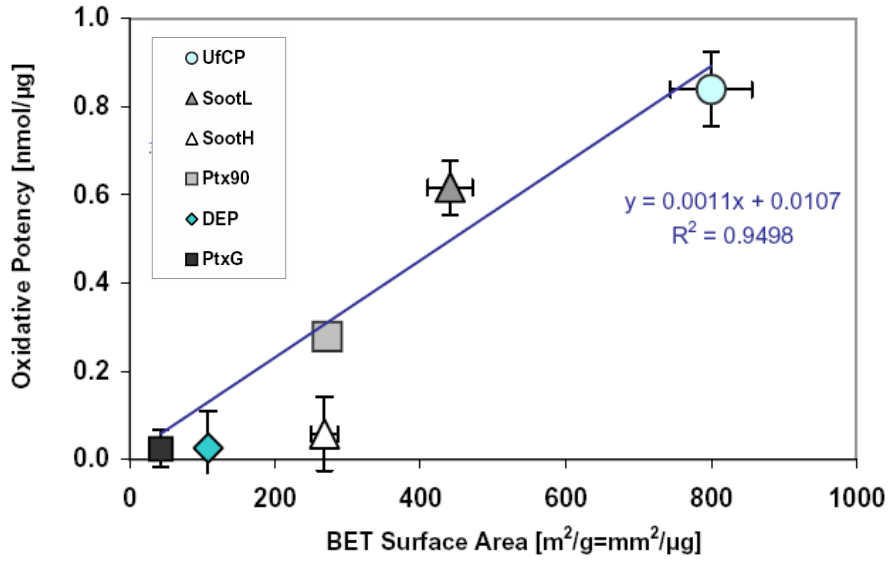


PHOTOCHEM quantiatation of antioxidative capacity

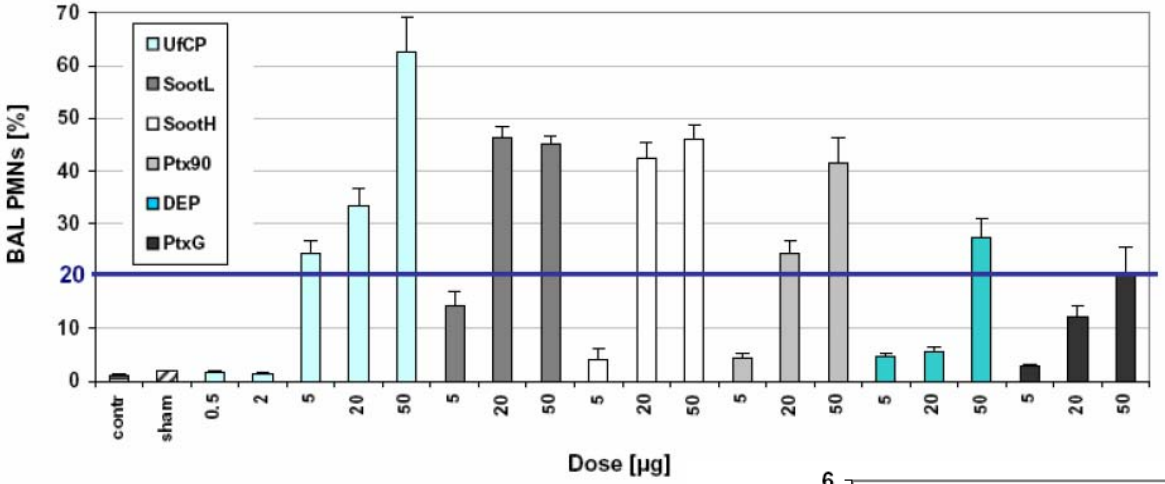
fast photochemical excitation of radical formation combined with sensitive luminometric detection

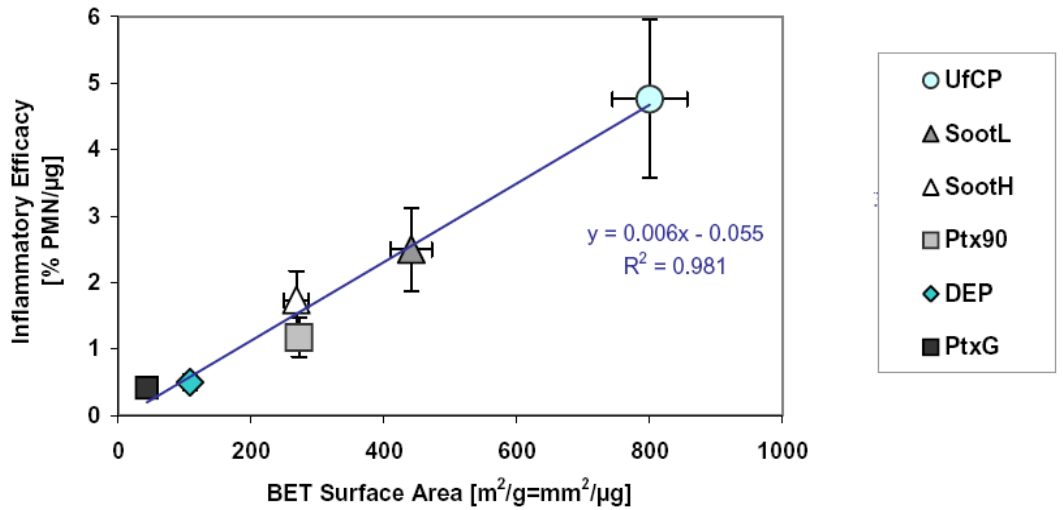
Oxidative Reactivity / Potency of Nanoparticles, A Function of their Surface Area?



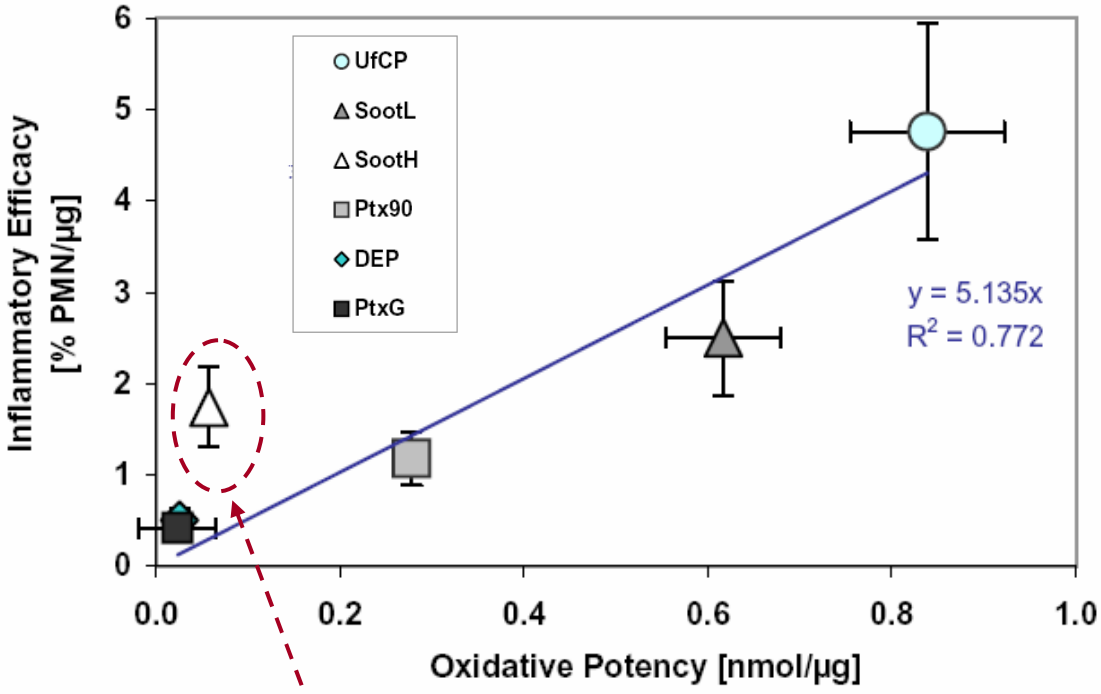


Inflammatory Efficacy of Nanoparticles, a Function of their Surface Area?





Can Oxidative Potency Predict the Inflammatory Efficacy of Nanoparticles?



NPs	Diameter [nm]	Org. Content [%]	BET surface [m^2/g]
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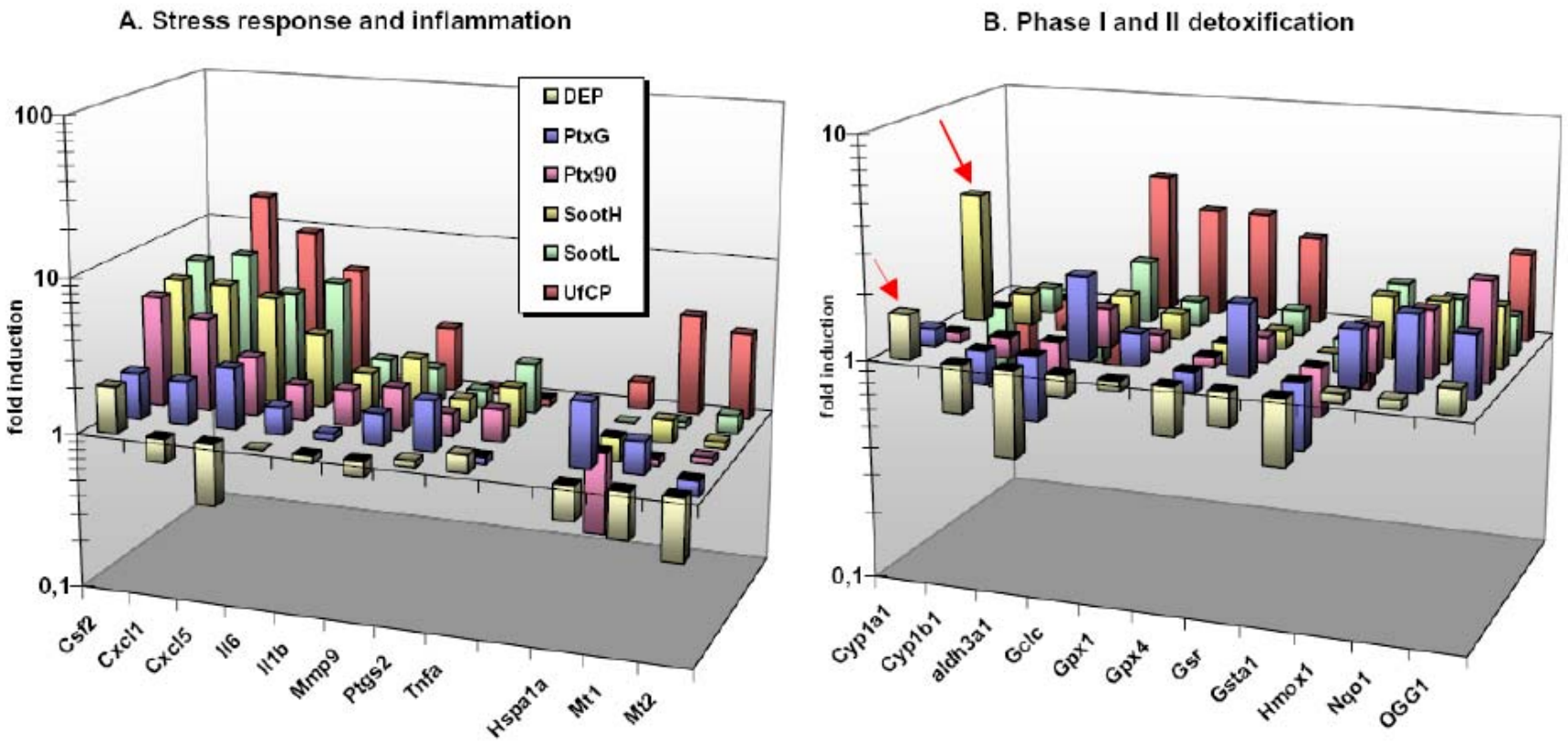
SootH	12	19	268
SootL	11	7	441
UfCP	10	<4	600

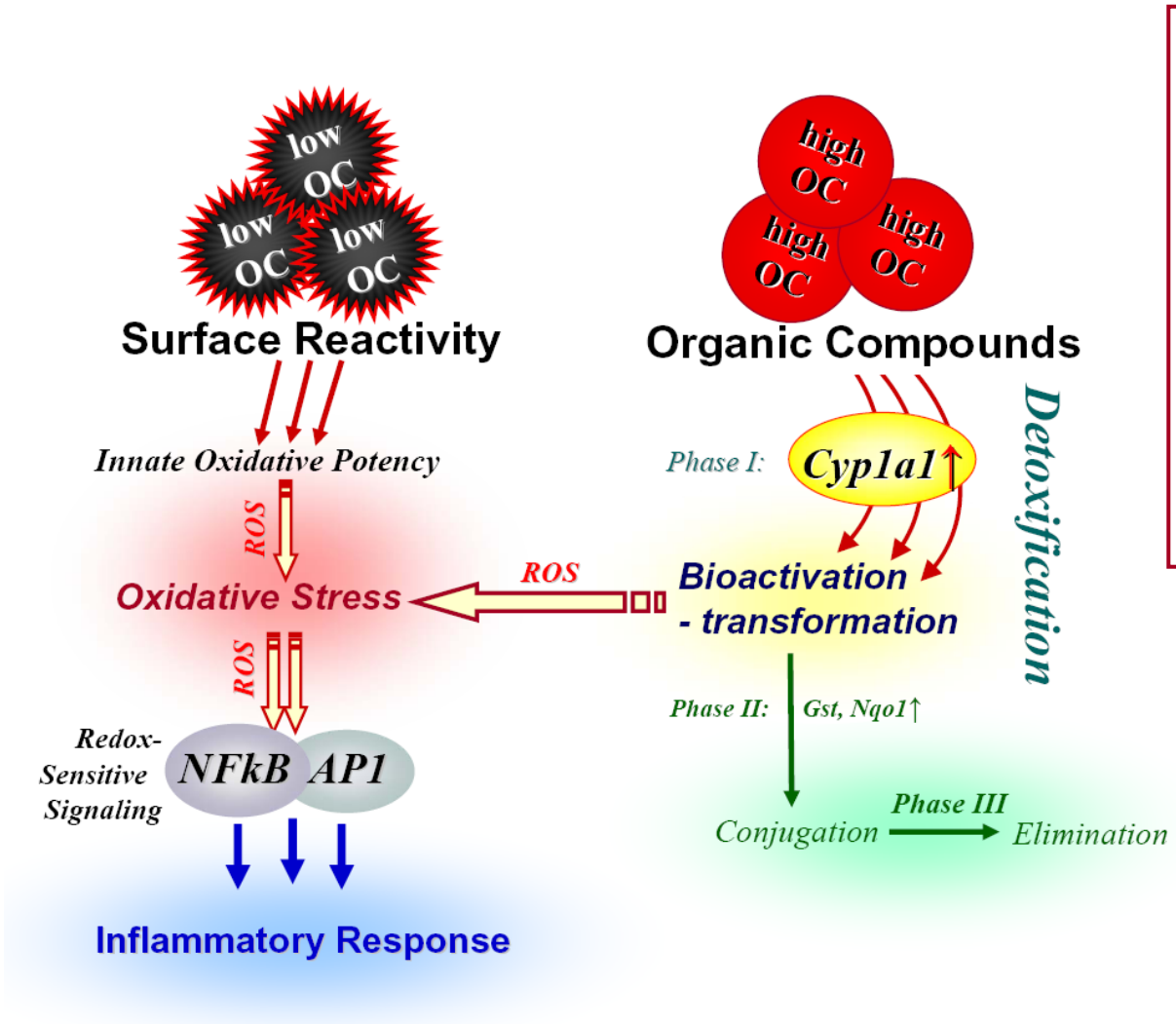
All but not *SootH* particles follow the relation! Why?

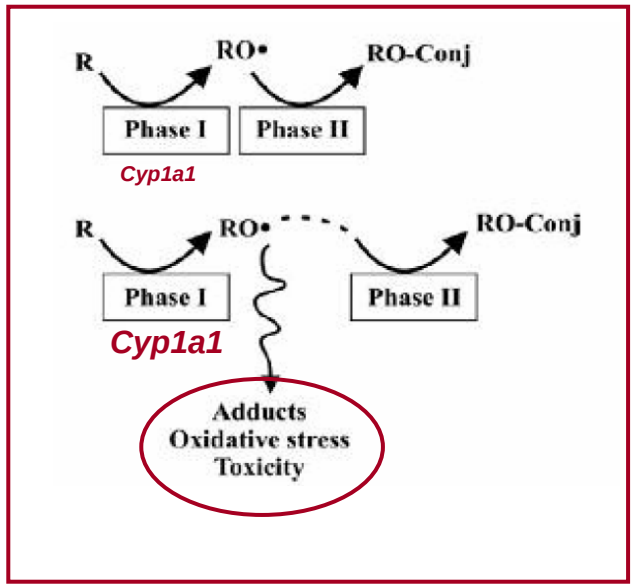
Bioavaliability of Organic Compounds Investigated by Gene Expression Analysis



Cyp1a1 expression matches well with the "Oxidative Potency" / " Inflammatory Efficacy" discrepancy

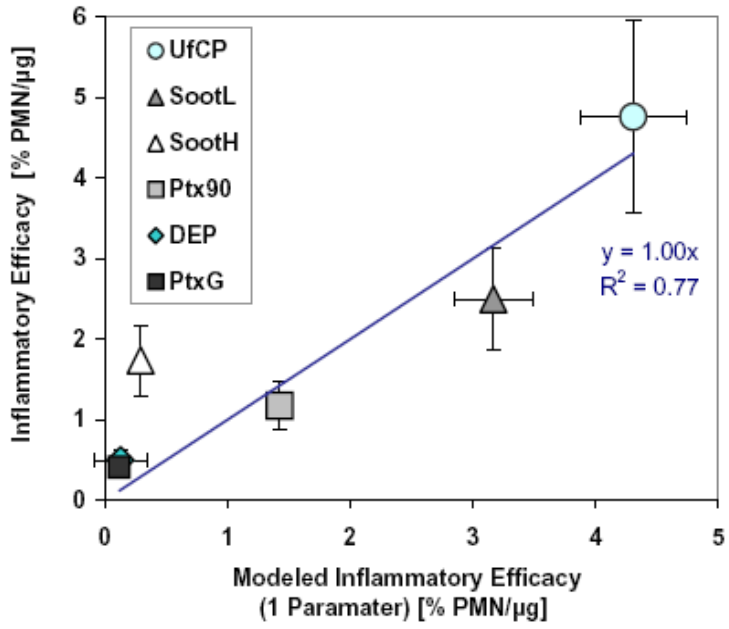
Pathways that Contribute to the Particle Induced Inflammatory Response





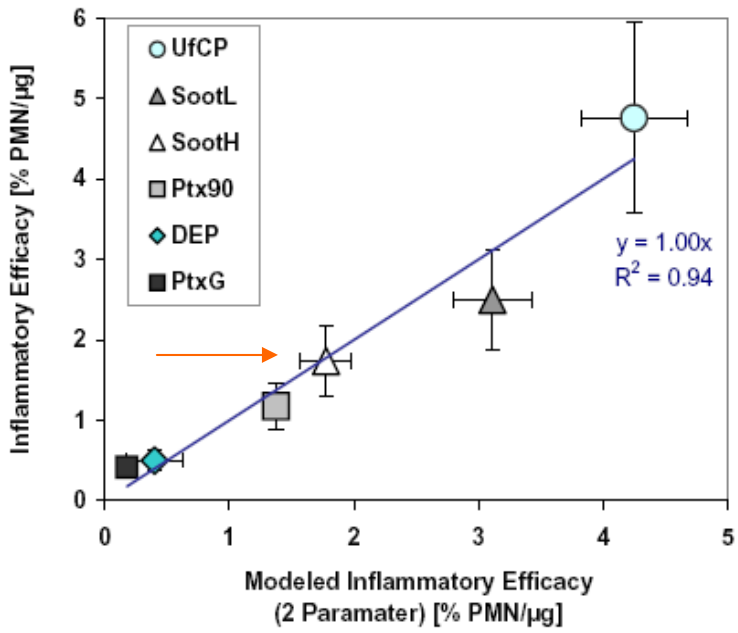
Quantitative Model for Inflammatory Efficacy: *A Two Pathway Concept*

Oxidative Potency = Surface Reactivity Only:



$$I_{Ef} = 5.14 Ox_{Pot}\left[nmol/\mu g\right]$$

Surface Reactivity + Metabolic Activation:



$$I_{Ef} = 5.05 Ox_{Pot}\left[nmol/\mu g\right] + 0.509\left(GE_{Cyp1a1} - 1\right)$$

Conclusions

- **Surface Toxicity is of major importance**
- **Toxicity of Carbonaceous Nanoparticles not only depending on organic contribution**

  SootL (1.7) even exceeds inflammatory efficacy of SootH (2.5)

  ⇒ ***Impact on toxicity of modern DEP (low OC high OxPot)?***

- **Toxicity or inflammatory efficacy can be predicted by a two parameter model that involves:**

  1. **Oxidative potency *(cell free assay)***
  2. ***Induction of Cyp1a1 gene expression***

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