The Impact of Porous Silicon Nanoparticles on Human Cytochrome P450 Metabolism in Human Liver Microsomes \textit{In Vitro}

Elisa Ollikainen\textsuperscript{a}, Dongfei Liu\textsuperscript{a}, Arttu Kallio\textsuperscript{a}, Ermei Mäkilä\textsuperscript{b}, Hongbo Zhang\textsuperscript{a,c}, Jarno Salonen\textsuperscript{b}, Hélder A. Santos\textsuperscript{a}, Tiina Sikanen\textsuperscript{a*}

\textsuperscript{a} Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland

\textsuperscript{b} Laboratory of Industrial Physics, Department of Physics and Astronomy, University of Turku, Turku, Finland*

\textsuperscript{c} Harvard John A. Paulson School of Engineering and Applied Sciences, Harvard University, Cambridge MA 02138, USA

*Corresponding author: Dr. Tiina Sikanen, Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, P.O. Box 56 (Viikinkaari 5E), FI-00014 University of Helsinki, Helsinki, Finland; Tel: +358-294159173; Email: tiina.sikanen@helsinki.fi
The Impact of Porous Silicon Nanoparticles on Human Cytochrome P450 Metabolism in Human Liver Microsomes In Vitro

Engineered nanoparticles are increasingly used as drug carriers in pharmaceutical formulations. This study focuses on the hitherto unaddressed impact of porous silicon (PSi) nanoparticles on human cytochrome P450 (CYP) metabolism, which is the major detoxification route of most pharmaceuticals and other xenobiotics. Three different surface chemistries, including thermally carbonized PSi (TCPsi), aminopropylsilane-modified TCPsi (APTES-TCPsi) and alkyne-terminated thermally hydrocarbonized PSi (Alkyne-THCPSi), were compared for their effects on the enzyme kinetics of the major CYP isoforms (CYP1A2, CYP2A6, CYP2D6, and CYP3A4) in human liver microsomes (HLM) in vitro. The enzyme kinetic parameters, $K_m$ and $V_{max}$, and the intrinsic clearance ($CL_{int}$) were determined using FDA-recommended, isoenzyme-specific model reactions with and without PSi nanoparticles. Data revealed statistically significant alterations of most isoenzyme activities in HLM in the presence of nanoparticles at 1 mg/mL concentration, and polymorphic CYP2D6 was the most vulnerable to enzyme inhibition. However, the observed CYP2D6 inhibition was shown to be dose-dependent in case of TCPsi and Alkyne-THCPSi nanoparticles and attenuated at the concentrations below 1 μg/mL. Adsorption of the probe substrates onto the hydrophobic Alkyne-THCPSi particles was also observed and taken into account in the determination of the kinetic parameters. Three polymer additives commonly used in pharmaceutical nanoformulations (Pluronics F68 and F127, and polyvinylalcohol) were also separately screened for their effects on CYP isoenzyme activities. These polymers had less effect on the enzyme kinetic parameters, and resulted in increased activity rather than enzyme inhibition, in contrast to the PSi nanoparticles. Although the chosen subcellular model (HLM) is not able to predict the cellular disposition of PSi nanoparticles in hepatocytes and thus provides limited information of probability of CYP interactions in vivo, the present study suggests that mechanistic interactions by the PSi nanoparticles or the polymer stabilizers may appear if these are effectively uptaken by the hepatocytes. Keywords: porous silicon; nanoparticles; drug metabolism; cytochrome P450; Pluronics
1 Introduction

Engineered nanoparticles are increasingly used in a variety of medical applications, including bioimaging, disease diagnoses, and drug delivery. Porous silicon (PSi) nanoparticles are a new class of nanocarriers used in drug delivery, and their feasibility for a range of medical uses is currently under evaluation (Salonen et al. 2008). The PSi nanoparticles have many advantages over the conventional drug carriers, including good biocompatibility both in vitro and in vivo (Bimbo et al. 2010, Korhonen et al. 2016, Nieto et al. 2013), the capability of enhancing the dissolution rates of poorly water-soluble payloads (Liu et al. 2013, Salonen et al. 2008, Santos et al. 2011), a high loading capacity (Liu et al. 2014, Salonen et al. 2005), and tunable surface chemistry (Huotari et al. 2013, Shahbazi et al. 2013, Shahbazi et al. 2014). Drug nanocarriers are by design intended for human use, thus their safety and toxicity to humans must be carefully evaluated prior to use. Common safety considerations of pharmaceutical nanoparticles typically address cytotoxicity, immune response, oxidative stress and production of reactive oxygen species, interactions with blood components, effects on the cardiovascular system, and biodegradation or accumulation in the body (De Jong et al. 2008, Naahidi et al. 2013). The influences of the nanoparticles on the molecular level biotransformation reactions of the co-administered drugs and other small molecular weight xenobiotics are however often neglected. For instance, the hepatic clearance of drugs may significantly change if the nanoparticles are capable of interacting with the enzymes that mediate drug detoxification, such as the hepatic cytochrome P450 (CYP) enzymes that are the major elimination pathway of drugs in humans (Anzenbacher and Anzenbacherová 2001).

The amount of PSi nanocarriers administrated is largely dependent on their drug loading degree and the purpose of use. Although the doses of intravenously
administered PSi nanoparticles are much lower than those of orally administered nanoparticles, these have been observed to accumulate in the liver and the spleen (Bimbo et al. 2010, Kallinen et al. 2014, Sarparanta et al. 2012, Wang et al. 2015a). However, the probability of the cellular uptake of PSi nanoparticles in hepatocytes is presently unknown. It also remains to be shown whether the PSi nanoparticles undergo vesicle transport into lysosomes or experience similar fate to drug molecules by interacting with the membrane-associated CYP enzymes located in the endoplasmic reticulum. Typically most nanoparticles are uptaken by the mononuclear phagocyte system, such as the Kupffer cells in liver or the macrophages in spleen, and it is known that both size and surface chemistry can impact the cellular uptake of nanoparticles in general (Huang et al. 2010). For instance, positive surface charge has been shown to favour uptake of gold nanoparticles by hepatocytes (Elci et al. 2016), and thus, also increase the risks of CYP interactions therein.

CYP-mediated metabolism is typically inhibited by competitive binding of the inhibitor to the enzyme’s active site, or elsewhere on the enzyme to inhibit its activity noncompetitively or uncompetitively (Pelkonen et al. 2008). Particularly, the broad substrate specificity of CYP3A4 (metabolizes 50% of clinically used drugs) and the genetic polymorphism associated with CYP2D6 (metabolizes 30% of clinically used drugs) may result in significant changes in the efficacy and safety of the treatment (Ingelman-Sundberg et al. 2007), if these isoenzyme activities are affected by the exogenous inhibitors (such as nanoparticles). On the other hand, even minor CYP enzyme inhibition may lead to excessive (toxic) drug concentrations for drugs that have very narrow therapeutic range and only one elimination pathway. In addition to enzyme binding, nanoparticles may interfere with the microsomal lipid bilayers (Raesch et al. 2015), and thereby affect the activity of human CYPs embedded in the inner membrane.
of mitochondria or in the endoplasmic reticulum (Anzenbauer and Anzenbauerová 2001). Some xenobiotics may also induce CYP gene expression on a cellular level, and thus, accelerate the metabolism of a drug compound and result in too low (insufficient) drug concentration in the body (Berg et al. 2007, Pelkonen et al. 2008). CYP profiling and interaction screening in vitro are therefore mandatory steps in the early phases of drug discovery (FDA 2014). Although prior studies have demonstrated that, for example, silver and gold nanoparticles may significantly affect CYP metabolism in vitro and in vivo (Midde and Kumar 2015), the impacts of drug nanocarriers, such as PSi nanoparticles, have not been examined before.

This study focuses on the hitherto unaddressed effects of PSi nanoparticles on the human phase I metabolism in human liver microsomes (HLM) in vitro mediated by CYP3A4 and CYP2D6 (major drug toxifying isoenzymes), CYP1A2 (activation of, e.g., carcinogenic polycyclic aromatic hydrocarbons (PAHs), and aflatoxin B₁) and CYP2A6 (endogenous and exogenous steroid regulation and detoxification of, e.g., nicotine and polychlorinated biphenyls) (Anzenbacher and Anzenbacherová 2001, McGraw and Waller 2006, Pelkonen et al. 2008). The PSi nanoparticle types were chosen so that they have different surface properties (surface charge, hydrophobicity) and chemistries: thermally carbonized PSi (TCPSi), aminopropylsilane-modified TCPSi (APTES-TCPSi), and alkyne-terminated thermally hydrocarbonized PSi (Alkyne-THCPSi). As detailed information on the distribution of PSi nanoparticles in the liver does not exist, we focused on determining the CYP isoenzyme inhibition by using the HLM. Although the chosen model (HLM) is not able to predict the cellular disposition of PSi nanoparticles in hepatocytes and thus provides only limited information of probability of CYP interactions in vivo, it provides an established and well-characterized platform for mechanistic enzyme inhibition studies in vitro. In HLM, the
CYPs are enriched, which provides a rapid approach to enzyme interactions screening besides the human liver cell lines, which may lose their liver-specific functions and thus show limited CYP activity upon culturing as such (Guillouzo et al. 2007, Liu et al. 2015). The inhibition effects of selected polymer stabilizers commonly used in nanoparticle formulations, namely polyvinyl alcohol (PVA) and block copolymers (Pluronics) F68 and F127 (Batrakova and Kabanov 2008, Sahoo et al. 2002), were also examined, as these may induce lipid bilayer rearrangements and thus affect the CYP activity for their part if effectively uptaken long with the nanoparticles into the hepatocytes. The impacts of PSi nanoparticles and polymers on CYP metabolism in HLM were examined using two independent analytical techniques, namely liquid chromatography-mass spectrometry (LC-MS) for determining the kinetics of FDA recommended CYP model reactions with and without PSi, and high-throughput well-plate (luminescence) assays for determining the minimum inhibitory concentrations of PSi nanoparticles.

2 Methods

2.1 Materials and Reagents

Acetonitrile, coumarin, dimethyl sulfoxide (DMSO), formic acid, furafylline, 6β-hydroxytestosterone (100 µg/ml in methanol), ketoconazole, magnesium chloride, methanol, 8-methoxypsoralen, β-nicotinamide adenine dinucleotide 2’-phosphate reduced tetrasodium salt hydrate (NADPH), paracetamol, phenacetin, polyvinyl alcohol, quinidine, testosterone, Trizma base and umbelliferone were from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid, potassium dihydrogenphosphate, and perchloric acid were from Riedel-deHaën (Seelze, Germany). Dipotassium hydrogenphosphate was from Amresco (Solon, Ohio, USA). Bufuralol was from Roche...
(Basel, Switzerland) and 1’-hydroxybufuralol from Toronto Research Chemicals (North York, ON, Canada). Pluronics F68 and F127 were from BASF (Florham Park, New Jersey, USA). Human liver microsomes (pooled from 20 donors, 20 mg/ml total protein content) were from Corning (Wiesbaden, Germany). Pierce BCA (bicinchoninic acid) Protein Assay Kit was from Thermo Fisher Scientific (Rockford, IL, USA). The P450-Glo™ CYP2D6 Assay kit was from Promega (Madison, WI, USA). Water was purified with a Milli-Q water purification system (Millipore, Molsheim, France).

2.2 Nanoparticle Preparation

Monocrystalline boron–doped p⁺-type Si <100> wafers with a resistivity of 0.01–0.02 Ωcm were used in the fabrication of the PSi nanoparticles. Multilayer PSi films were prepared by electrochemically etching the Si wafers in a 1:1 (v/v) aqueous hydrofluoric acid (38%)–ethanol electrolyte with a pulsed etching profile. Free-standing films were obtained by increasing the etching current to the electropolishing region. PSi films of three different surface chemistries were crafted: TCPSi (Salonen et al. 2005), APTES-TCPSi (Liu et al. 2015, Mäkilä et al. 2012), and Alkyne-THCPSi (Wang et al. 2015b). The size reduction of the obtained multilayer films to nanoparticles was accomplished by wet milling. The final size selection of the PSi nanoparticles was done by centrifugation (16 000 g, 10 min).

2.3 Nanoparticle Characterization

The porous properties of the produced nanoparticles were characterized by nitrogen adsorption/desorption measurements on a Tristar 3000 (Micromeritics Inc., USA) at 77 K. The specific surface area was calculated using the Brunauer–Emmett–Teller method, and the pore volume was determined from the isotherm using the total adsorption value at relative pressure p/p₀ = 0.97. Particle size and surface zeta (ζ)-potential measurements
were performed on a Zetasizer Nano ZS (Malvern Instruments Ltd., UK). The PSi nanoparticles were dispersed in an aqueous solution with a pH value of 7.4. Both the size and ζ-potential were recorded as the mean of three measurements.

The morphologies of the fabricated PSi nanoparticles were evaluated by transmission electron microscope (TEM; Tecnai 12, FEI Company, USA) at an acceleration voltage of 120 kV. The TEM samples were prepared by depositing the PSi nanoparticle ethanol suspensions (2 μL, 20 μg/mL) onto carbon-coated copper grids (300 mesh; Electron Microscopy Sciences, USA). The grids were then air-dried prior to imaging. The chemical composition analysis of the PSi nanoparticles was performed by Fourier transform infrared spectroscopy as described elsewhere (Liu et al. 2015, Mäkilä et al. 2012, Salonen et al. 2002, Wang et al. 2015b).

2.4 Enzyme Incubations

All enzyme incubations were carried out by using HLM. The isoenzyme specific model reactions for enzyme kinetic studies by LC-MS were selected according to FDA recommendations (2012, 2014) and were phenacetin O-deethylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), bufuralol 1’-hydroxylation (CYP2D6), and testosterone 6β-hydroxylation (CYP3A4) (Figure S1). The minimum inhibitory concentration of nanoparticles was determined only with CYP2D6 using Luciferin-based model reaction (Figure S3). The CYP probe substrates were diluted in the incubation buffer, except for testosterone, which did not dissolve in purely aqueous solutions, but required the addition of 50% (v/v) methanol in all dilutions (5% v/v in the reaction mixture).

The enzymatic reactions were performed in a total volume of 100 μl. The incubation time, the buffer, the substrate and the HLM concentrations were adjusted enzyme-specifically according to the manufacturer’s instructions as described in the
Supplementary material (Tables S1 and S6). The incubation conditions of CYP2A6 were optimized in-house to enable determination of Michaelis-Menten kinetics. After the addition of the substrate and the HLM, the reaction mixture was preincubated at 37°C for 5 min before initiating the reaction by the addition of NADPH (1 mM in reaction). The reaction mixture was incubated at 37°C for the specified time after which the reaction was terminated by adding 10 µl of 4M ice-cold perchloric acid (enzyme kinetic assays by LC-MS) or 100 µl Luciferin detection reagent with esterase (enzyme inhibition assays by luminescence). The LC-MS samples were then placed on ice for 10 min, after which the HLM precipitate was removed by centrifugation (16 000 g, 10 min) and the supernatant was analyzed as such or diluted 1:1 (v/v) with the buffer (supernatants of the CYP2D6 reaction). The luminescence samples were allowed to stand still for 20 min prior to analysis by Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Rockford, IL, USA).

2.5 Enzyme Kinetic Studies

Effects of three PSi nanoparticle types (1 mg/ml in the reaction, 1.25-2.5 mg/mg total protein) and of the selected three polymers (0.1%, m/v, in the reaction) on the enzyme kinetics of the CYP model reactions were studied with and without the nanoparticles and the polymers. The reactions were performed as described above. The nanoparticle suspension (10 mg/ml in water) or polymer solution (1%, m/v, in water) were added to the reaction solution before the preincubation step. After the reaction, the nanoparticles were removed from the sample by centrifugation (16 000 g, 10 min) (simultaneously to the HLM precipitate). The Michaelis constants (K_m) and enzyme activities (V_max) of the selected model reactions were determined using seven different substrate concentrations (each in duplicate) and blank controls without the substrate. GraphPad Prism 5 software
was used to fit the data to the nonlinear Michaelis-Menten model and to calculate the $K_m$ and $V_{max}$ values. The intrinsic clearances ($CL_{int}$) were calculated as the ratio of $V_{max}$ and $K_m$. The Student’s $t$-test with Welch’s correction was performed for statistical analysis of the observed differences in the enzyme kinetic parameters, $K_m$, $V_{max}$, and $CL_{int}$. In addition, the inhibition mechanisms were also examined using linear regression Lineweaver-Burk plots (Figure S2).

2.6 Determination of the minimum inhibitory concentration of nanoparticles

The effect of nanoparticle dose on CYP2D6 inhibition was also studied using P450-Glo™ Assay at seven different concentrations (1 ng/ml-1 mg/ml) of each nanoparticle type (Table S6). The results were compared to control incubations without nanoparticles.

2.7 Adsorption of Probe Substrates and Metabolites onto Nanoparticles

The role of nonspecific adsorption of probe substrates and metabolites was ascertained to distinguish it from enzyme inhibition by incubating the substrates and metabolites with the nanoparticles (1 mg/ml) without the enzymes. The concentrations of the substrates and metabolites were 500 nM (each) for bufuralol and 1’-hydroxybufuralol, and 2 µM (each) for other compounds. The incubations (n=2 each) were carried out at room temperature in the specified incubation buffer and for equally long as the CYP incubation time. After the specified incubation time, the nanoparticles were removed by centrifugation (16 000 g, 10 min) and the supernatants were analyzed. The peak areas were compared to the control samples without nanoparticles to calculate the percentage of the nonadsorbed (% free fraction) substrate and metabolite. The strong adsorption of bufuralol onto all nanoparticle types and of testosterone onto Alkyne-THCPSi nanoparticles necessitated that the adsorbed amount of these
compounds and their metabolites onto the nanoparticles were further determined at each different substrate concentration used in the enzyme kinetic studies. Metabolite concentrations were selected so that they corresponded to the amounts of metabolite formed in the control incubations at the respective substrate concentrations. The nanoparticle concentration was 1 mg/ml and the same protocol was followed in these experiments. The percentages of unbound substrate and metabolite were taken into account to correct the enzyme kinetic parameters to distinguish the enzyme inhibition from adsorption effects.

2.8 Interactions between the Nanoparticles and Human Liver Microsomes

The nonspecific interactions of the nanoparticles with the HLM incorporating the CYP enzymes were examined by two different protocols to characterize the supernatant and the precipitate (the nanoparticles) after incubation of the nanoparticles with the HLM in both buffers.

The total free (unbound) protein in the supernatant was quantified using a commercial BCA based colorimetric method. The nanoparticles (1 mg/ml) were incubated with HLM (0.4-0.8 mg/ml total protein amount) in a total volume of 100 µl at 37°C for 25 min. After incubation, the nanoparticles were removed by centrifugation (16,000 g, 10 min) and the supernatant was analysed according to supplier’s instructions following the microplate procedure (sample to working reagent ratio 1:8). The standard curve was, however, prepared using the total protein content of the HLM as a reference instead of bovine serum albumin to enable a more accurate quantification of the HLMs’ protein content in the presence of microsomal lipids. Briefly, 25 µl of supernatant was mixed with 200 µl of freshly prepared working reagent and incubated for 30 min at 37°C. After cooling to room temperature, the UV-absorbance at 562 nm was measured by Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Rockford, IL, USA).
The obtained values were compared to those of control samples prepared in a similar manner, but without nanoparticles. All BCA assays were done in triplicate.

The nanoparticles were characterized after incubation using different HLM quantities in different buffers, and the PSi nanoparticles were imaged by scanning electron microscopy (SEM, Zeiss Super 55). The controls were prepared in the same buffers without the enzyme.

The zetapotential of the HLM was also determined using Zetasizer Nano ZS (Malvern Instruments Ltd., UK). The HLM stock was diluted at the ratio of 1:25 with the Milli-Q water before the measurement.

### 2.9 Analytical Method Development and Validation

Apart from luminescence (CYP2D6 inhibition) and BCA colorimetric assays, all samples were analyzed by liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) in positive ion mode using an ACQUITY UPLC™ chromatograph (Waters, Milford, MA, USA) and a Xevo Q-TOF mass spectrometer (Waters, Manchester, UK). The chromatographic parameters, the solvent gradient and the MS parameters are given in Tables S2–S4. Data were analyzed by MassLynx V4.1 software.

The analytical method was validated for specificity, linearity, limits of detection and quantitation, and repeatability according to ICH guidelines (Table S5). The metabolites were diluted either in buffer (to the limits of detection and quantitation) or in the blank incubation matrix (the determination of the dynamic range and linearity, and the repeatability) for method development and validation. Specificity of the method was determined using blank samples, *i.e.*, incubation matrix not containing NADPH,
substrate or HLM, or terminated by adding acid before the substrate (zero reaction time).

3 Results

3.1 Nanoparticle characterization

Both APTES-TCPSi and TCPSi modifications resulted in hydrophilic surface, whereas Alkyne-THCPSi nanoparticles were hydrophobic (Table 1). The characterization of PSi nanoparticles by transmission electron microscopy (TEM) revealed the irregular shapes of all nanoparticle types regardless of their surface modification (Figure 1a). The mean particle sizes, confirmed by dynamic light scattering, were similar for all nanoparticle types: ca. 159, 176, and 184 nm for TCPSi, APTES-TCPSi, and Alkyne-THCPSi, respectively (Figure 1b). The amine-modified APTES-TCPSi nanoparticles were positively charged (ca. +35 mV), whereas the other two PSi nanoparticle types had negative surface charge of ca. –30 mV (Figure 1c). The pore volumes for the different PSi nanoparticles were somewhat similar (ca. 0.5-0.7 cm³ g⁻¹) and the pore diameters were within 8-12 nm (Table 1).

3.2 Adsorption of Small Molecules onto Porous Silicon Nanoparticles

The direct interactions between the nanoparticles and the selected isoenzyme specific probe substrates and metabolites were determined upon incubation without the HLM to distinguish between molecular adsorption effects and enzyme inhibition (Figure S4). All selected probe substrates and metabolites were shown to be fairly compatible for CYP interaction screening in the presence of the TCPSi particles, as no significant adsorption was observed. Only in the case of bufuralol was the free fraction less than 75%, whereas its specific metabolite, 1'-hydroxybufuralol, had over 125% yield in the
presence of TCPSi nanoparticles. The APTES-TCPSi particles were similarly compatible with most metabolites and the probe substrates phenacetin (CYP1A2) and testosterone (CYP3A4). However, the free fraction of bufuralol was again less than 50% and that of 1’-hydroxybufuralol over 125%. Some adsorption of coumarin (free fraction <75%) onto APTES-TCPSi nanoparticles was also observed. The hydrophobic Alkyne-THCPSi particles adsorbed most of the selected probe substrates as expected. The free fraction of bufuralol was less than 6%. Instead the metabolites, which are generally more hydrophilic, did not adsorb as substantially onto the Alkyne-THCPSi particles, except for 1’-hydroxybufuralol and 6β-hydroxytestosterone (free fractions ca. 60% each). When adsorption was further studied with different substrate and metabolite concentrations, we observed that the relative adsorption of bufuralol and 1’-hydroxybufuralol onto all nanoparticle types, and testosterone onto Alkyne-THCPSi nanoparticles were generally more pronounced at small concentrations until the nanoparticles’ surfaces became saturated with the nonspecifically bound substrates and/or metabolites (Figure S5). The free fractions (%) of unbound substrate and metabolite were taken into account to correct the enzyme kinetic parameters.

3.3 The Impact of Porous Silicon Nanoparticles on Intrinsic Clearances and Enzyme Kinetics

All three types of nanoparticles (1.25 mg/mg total protein) caused decrease in $V_{\text{max}}$ of phenacetin $O$-deethylation by CYP1A2, which indicated enzyme inhibition. Decrease in $K_{\text{m}}$ also occurred which indicated improved affinity (Figure 2a,e). These findings suggest inhibition by noncompetitive (inhibitor binds outside the enzyme’s active site) or uncompetitive (inhibitor binds to the enzyme-substrate complex) mechanisms rather than by competitive inhibition (Berg et al. 2007). However, as a whole, addition of PSi nanoparticles resulted in increased intrinsic clearances (CL$_{int}$) compared with the
control, particularly in the case of APTES-TCPSi and Alkyne-THCPSi particles (Figure 2i).

Coumarin 7-hydroxylation by CYP2A6 was also affected by all nanoparticle types (2.5 mg/mg total protein) (Figure 2b,f). A minor increase in $V_{\text{max}}$ was observed in the presence of APTES-TCPSi, whereas TCPSi did not affect the $V_{\text{max}}$, but a decrease in $K_{m}$ was observed indicating improved enzyme affinity. Although these alterations in the kinetic parameters were statistically significant, the findings did not indicate enzyme inhibition, but rather increased intrinsic clearance in the presence of APTES-TCPSi and particularly, of TCPSi, particles (Figure 2j). In contrast, Alkyne-THCPSi nanoparticles resulted in prominent enzyme inhibition (decreased $V_{\text{max}}$), probably by noncompetitive mechanism as the $K_{m}$ of the reaction or the intrinsic clearance were not affected.

CYP2D6 was found the most prone to enzyme inhibition. A clear decrease in $V_{\text{max}}$ was observed in the presence of each type of PSi nanoparticles (1.25 mg/mg total protein) (Figure 2c,g). This eventually resulted in slightly reduced intrinsic clearances in the presence of all nanoparticles types tested (Figure 2k). Even after the adsorption of bufuralol was taken into account, an apparent decrease in $V_{\text{max}}$ relative to the control values was observed, though the changes in the intrinsic clearances were attenuated.

Similar inhibition (decreased $V_{\text{max}}$) of testosterone 6β-hydroxylation by CYP3A4 was also observed in the presence of APTES-TCPSi and TCPSi nanoparticles (2 mg/mg total protein). Instead, Alkyne-THCPSi nanoparticles resulted in clearly increased $V_{\text{max}}$, but also a somewhat increased $K_{m}$, which indicated competitive enzyme inhibition (Figure 2d,h). Again, adsorption correction did not significantly change the trend, but resulted in negligible change in the intrinsic clearance (Figure 2l). Only in case of TCPSi particles, the intrinsic clearance was slightly decreased compared to control.

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In all, the nanoparticles mainly reduced the intrinsic clearances of CYP2D6 and CYP3A4, while CYP2D6 was clearly the most vulnerable to inhibition by all nanoparticle types tested. Therefore, we confirmed the result by determining the minimum inhibitory concentrations of PSi nanoparticles by using a complementary, Luciferin-based CYP2D6-specific model reaction and luminescence-based quantitation. As a result, similar decrease in CYP2D6 activity (50-80% of the maximum) was observed with the complementary technique (Figure 3). Further study with seven different nanoparticle concentrations (down to 2.5 ng/mg total protein) showed that inhibition by APTES-TCPSi was in the same level (80% of the maximum) with all studied concentrations, whereas inhibition by TCPSi and Alkyne-THCPSi was more dose-dependent. TCPSi nanoparticles inhibited CYP2D6 with concentrations of 2.5 µg/mg total protein or higher, and Alkyne-THCPSi with concentrations of 25 µg/mg total protein or higher.

3.4 The Effects of Incubation Buffers and Nonspecific Interactions of the Human Liver Microsomes with the Nanoparticles

The SEM images of the nanoparticles after incubation with and without HLM in the different incubation buffers revealed some traces of adsorbed lipid membranes on all nanoparticle types (Figure S7). The BCA assay was able to distinguish some variation in the unbound fraction of HLM between the different nanoparticle types: TCPSi nanoparticles adsorbed less HLM than APTES-THCPSi, and substantially less than Alkyne-THCPSi. However, according to the SEM images, the Tris and phosphate ions also effectively penetrated the pores of the PSi nanoparticles, which is likely to play a role in terms of the lipid interaction as well. The BCA assay analysis of the unbound HLM revealed that the penetration of the negatively charged phosphate ions into the nanoparticles’ pores slightly reduced the interactions between the PSi nanoparticles and
the negatively charged HLM (zeta potential *ca.* -44±5 mV) (Figure S6). The positively charged Tris ions probably resulted in an accumulation of positive charges on the nanoparticles, and thus, somewhat greater interactions were observed with the negatively charged HLM in the Tris buffer.

### 3.5 The Impact of Polymer Additives on Intrinsic Clearances and Enzyme Kinetics

The CYP interactions of three pharmaceutical polymer additives (block copolymers F68 and F127, and PVA) commonly used in nanoparticle formulations were examined. Our study showed that apart from CYP3A4, the addition of the block copolymers F68 and F127 generally resulted in increased intrinsic clearances either *via* increased enzyme activity (CYP1A2 and CYP2A6) or increased enzyme affinity (CYP2D6), while PVA did not have any significant effect on the metabolic clearances of any of the isoenzymes (Figure 4). CYP2D6 was again the most prone to enzyme inhibition by the studied polymers, similarly as was observed in the case of the PSi nanoparticles.

### 4 Discussion

Apart from only a few studies on the impact of silver and gold nanoparticles on CYP metabolism in human and rat liver microsomes, on recombinant CYP enzymes and in rats *in vivo* (Kulthong *et al.* 2012, Sereemaspun *et al.* 2008, Ye *et al.* 2014), little is known about the impact of nanoparticles on CYP metabolism. The probability of cellular uptake of the PSi nanoparticles by the hepatocytes remains unknown and thus the impact of PSi nanoparticles has hardly been investigated until the present study. In this study, we chose to use HLM to examine the intrinsic clearances and the enzyme kinetics of CYP metabolism in the presence of PSi nanoparticles *in vitro*, although the
subcellular fractions generally allow only limited possibilities to predict the *in vivo* effects and no information on the probability of enzyme induction. However, compared with intact hepatocytes, the HLM provide a faster approach to examine the potential for mechanistic inhibition and activation.

We found statistically significant inhibition even though the PSi nanoparticles studied were relatively large (range of 160-180 nm). The earlier studies on gold and polymer nanoparticles (Fröhlich *et al.* 2010, Ye *et al.* 2014), suggest that smaller nanoparticles may have even greater impact on CYP metabolism as they may more easily enter the enzymes’ active site cavities, which are typically below 3 nm³ (Gay *et al.* 2010). PSi nanoparticles are often designed to adsorb small (drug) molecules. Consequently, they may interfere not only with the quantitative determination of the substrates and the metabolites, but also effectively bind to enzymes, enzyme-substrate complexes or lipids, which may result in uncompetitive or noncompetitive enzyme inhibition. This study shows that the enzyme inhibition caused by the PSi nanoparticles were mostly reversible in nature and due to a combination of competitive, noncompetitive and uncompetitive mechanisms (for comparison of the inhibition mechanisms, see Figure S2). The inhibition effects caused by the relatively large PSi nanoparticles in our study may thus be explained by the fact that the nanoparticles also interacted with the HLMs upon incubation without any substrate, as evidenced by adsorption of lipids on nanoparticles in SEM images and by changes in the total free (unbound) protein content determined by the BCA method. However, the buffer salts also penetrated into the pores of the PSi and were shown to have even greater effect on the amount of nonspecifically bound HLM than the native surface charge of the PSi particles. Namely, accumulation of the positively charged Tris ions on the PSi surface seemed to increase the electrostatic interaction with the negatively charged HLMs resulting in much greater
loss of free protein than that observed upon incubation of PSi particles with the negatively charged phosphate ions. At the same time, the differences between the different particles were subtle showing most interaction of HLM toward the hydrophobic Alkyne-THCPSi particles. It should be noted, though, that the BCA assay has only limited capability to quantify proteins in the presence of lipids, such as microsomal membranes, and thus, such small differences between the different nanoparticle types may also be the result of inter-assay variation. The trend was, however, similar in both incubation buffers tested.

The above findings on the HLM interactions with the PSi nanoparticles are, however, not capable of explaining all alterations observed in enzyme kinetics. For the model reactions, the incubation buffer were selected according to HLM supplier’s recommendations. Tris buffer, which seemed to increase the interaction between the HLM and PSi was used for determination of CYP1A2 and CYP2A6 isoenzyme activities, which had generally less interferences than those of CYP2D6 and CYP3A4 that had been determined upon incubation in phosphate buffer. Thus, the nonspecific HLM adsorption onto the PSi nanoparticles in different buffers did not directly correlate with the observed enzyme inhibition in each case, but other modes of interaction should also be considered. Our results also suggest that when evaluating the impacts of nanoparticles on CYP metabolism, it is not enough to determine only the alterations in enzyme activity ($V_{\text{max}}$), but the alterations in the enzyme affinity ($K_m$) should also be considered as these enable examination of the potential inhibition mechanisms, i.e., competitive binding to the active site (increased $K_m$) versus noncompetitive binding outside the active site (decrease in $V_{\text{max}}$, changes in $K_m$ are possible) and uncompetitive binding to the enzyme-substrate complex (decrease of both $V_{\text{max}}$ and $K_m$) (Berg et al. 2007). It was also shown that despite of significant decrease in the enzyme activity
caused by the PSi nanoparticles (like in the case of CYP2D6), the intrinsic clearances were much less affected due simultaneous increase in the enzyme affinity (decreased K_m).

Since relatively high PSi particle concentration (1 mg/mL) was used in the enzyme kinetic studies, a complementary spectroscopic technique was used to carry out a dose-dependency study to confirm the observation of the significantly reduced enzyme activity in case of CYP2D6. As a result, the CYP2D6 activity in the presence of APTES-TCPSi nanoparticles was reduced to 80% of the control independent of the nanoparticle concentration. The TCPSi and Alkyne-THCPSi nanoparticles caused more dose-dependent CYP2D6 inhibition attenuating at concentrations of 0.1 and 1 μg/mL, respectively. These results suggest that mechanistic enzyme inhibition may occur in the presence of PSi nanoparticles if these are effectively uptaken by the hepatocytes. However, since the results were derived using subcellular fractions only, further studies on the nanoparticles’ impact on CYP metabolism and on the degree of nanoparticles’ uptake in hepatocytes are required to be able to predict the impact of PSi nanoparticles in vivo in full. Furthermore, the combined effects of polymer stabilizers in pharmaceutical nanoformulations should also be considered as they may either compensate or further enforce the inhibition effects caused by the PSi nanoparticles if the polymer stabilizers are equally effectively uptaken by the cells. For example, in this study, the Pluronic block copolymers F68 and F127 clearly increased the metabolic clearance of CYP1A2, CYP2A6 and CYP2D6, which may overrule the inhibition effects caused by the nanoparticles.

5 Conclusions

We investigated the impacts of three differently modified PSi nanoparticles on the
intrinsic clearances and activities of four CYP enzymes by comparing the enzyme kinetic parameters with and without the nanoparticles in HLM *in vitro*. The CYP2D6 isoenzyme was the most vulnerable to enzyme inhibition by the PSi nanoparticles, as its remaining activities ($V_{\text{max}}$) were only 40-62% of the controls regardless of the nanoparticle type. However, the simultaneous increase in enzyme affinity ($K_m$) resulted in less significant changes in intrinsic clearances ($CL_{\text{int}}$) apart from the APTES-TCP Si nanoparticles. A further study with seven nanoparticle concentrations confirmed that APTES-TCP Si concentrations as low as 2.5 ng/mg total protein reduced the activity of CYP2D6 to 80% of the control independent of the nanoparticle concentration. The effects of the other two nanoparticle types were more dose-dependent and attenuated at concentrations which are closer to those that might exist in liver *in vivo*. However, direct extrapolation of these findings, obtained using subcellular fractions, to *in vivo* situation should be treated with care as the HLM does not provide any information on the probability of the cellular uptake of the PSi nanoparticles by hepatocytes. Taking into account the substantial genetic polymorphism associated with CYP2D6, the observed 20% decline in enzyme activity *in vitro* may be surpassed by the large variation in the enzyme activity *in vivo*.

The effects of three pharmaceutical polymer additives (block copolymers F68 and F127, and PVA) on CYP metabolism were also examined. CYP2D6 was again the most prone to enzyme inhibition by all of the polymers. However, thanks to the increased enzyme activities, the F68 and F127 polymers rather increased and PVA had no significant effect on the intrinsic clearances.

Our data show that statistically significant CYP interactions may result in an *in vitro* situation from the use of PSi nanoparticles as drug nanocarriers. In contrast to previous research, this study confirmed that even relatively large nanoparticles (in the
range of 160-180 nm) may inhibit the CYP enzyme activities in vitro. In addition, the observed enzyme inhibition caused by the nanoparticles is probably a combination of the different inhibition mechanisms (competitive, noncompetitive and uncompetitive) as well as other interactions (such as nonspecific adsorption of lipids or electrostatic interactions with salts). The use of polymer stabilizers may further complicate the scheme. Therefore, further studies on the probability of cellular uptake of PSi nanoparticles and CYP interaction screening using hepatocytes are required in order to better predict the in vivo response. Nevertheless, our results highlight the need for more comprehensive investigations of the CYP interactions caused by PSi nanoparticles, as inhibition (or induction) of the CYP activities may alter the elimination of drugs and other xenobiotics in vivo if the nanoparticles are effectively uptaken by the hepatocytes.

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References


**Appendices**

The Supplementary material includes detailed experimental conditions, structures of the probe substrates and their metabolites, analytical method validation results, results of the adsorption studies (adsorption of the probe substrates, metabolites and HLM on the nanoparticles), and linear Lineweaver-Burk plots for enzyme kinetics.
Table(s) with caption(s)

Table 1. Properties of the PSi nanoparticles used in the study (mean and standard deviation).

<table>
<thead>
<tr>
<th>Nanoparticle type</th>
<th>TCPSi</th>
<th>APTES-TCPSi</th>
<th>Alkyne-THCPSi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pore Diameter (nm)</td>
<td>10.7</td>
<td>8.0±0.7</td>
<td>12.1±1.2</td>
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<tr>
<td>Pore Volume (cm$^3$g$^{-1}$)</td>
<td>0.50±0.01</td>
<td>0.69±0.07</td>
<td>0.53±0.01</td>
</tr>
<tr>
<td>Specific surface area (m$^2$g$^{-1}$)</td>
<td>184±4</td>
<td>345±3</td>
<td>176±14</td>
</tr>
<tr>
<td>Hydrophilicity</td>
<td>Hydrophilic</td>
<td>Hydrophilic</td>
<td>Hydrophobic</td>
</tr>
</tbody>
</table>
Figures

Figure 1. a) Characterization of the prepared PSi nanoparticles by TEM. b) Size distribution and c) surface charge of the PSi nanoparticles.
Figure 2. Relative (% of control) maximum reaction rates ($V_{\text{max}}$), the Michaelis constants ($K_{\text{m}}$) and the intrinsic clearances ($\text{CL}_{\text{int}}$) of the four CYP model reactions in the presence of the PSi nanoparticles. The control values of $V_{\text{max}}$ (without nanoparticles) are adjusted to 100%. The error bars show the standard deviations ($n = 4$). The values after adsorption correction are marked by (a) after the name of the nanoparticle on the x-axis. The results of Student’s $t$-test with Welch’s correction are presented above the bars as follows (each value compared to control): *** = $p<0.001$; ** = $0.001<p<0.01$; * = $0.01<p<0.05$; ns = not significant, $p>0.05$. # The Michaelis-Menten fit was not able to reliably predict the $K_{\text{m}}$ value, which prevented the calculation of the $\text{CL}_{\text{int}}$. 
Figure 3. Inhibition of CYP2D6 by (a) TCPSi, (b) APTES-TCPSi, and (c) Alkyne-THCPSi nanoparticles relative to the control with different nanoparticle concentrations. The control activity (without nanoparticles) is adjusted to 100% (dash line).
Figure 4. Relative (%) maximum reaction rates ($V_{\text{max}}$), the Michaelis constants ($K_m$) and the intrinsic clearances ($\text{CL}_{\text{int}}$) of the four CYP model reactions in the presence of the polymers. The control values of $V_{\text{max}}$ (without polymers) are adjusted to 100%. The error bars show the standard deviations ($n = 4$). The results of Student’s t-test with Welch’s correction are presented above the bars as follows (each value compared to control): *** = $p<0.001$; ** = $0.001<p<0.01$; * = $0.01<p<0.05$; ns = not significant, $p>0.05$. 
Interactions with microsomes

PSi nanoparticles

NADP⁺ → NADPH

CPR

P450

O₂ → RH → ROH → H₂O

Inhibition of P450 activity

Graphical Abstract (for review)