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### **Angaben zur Veröffentlichung / Publication details:**

Westerhausen, Christoph, Florian G. Strobl, Rudolf Herrmann, A. T. Bauer, S. W. Schneider, Armin Reller, Achim Wixforth, and Matthias F. Schneider. 2012. "Chemical and mechanical impact of silica nanoparticles on the phase transition behavior of phospholipid membranes in theory and experiment." *Biophysical Journal* 102 (5): 1032–38. <https://doi.org/10.1016/j.bpj.2011.12.004>.

## ***Chemical and mechanical impact of silica nanoparticles on the phase transition behavior of phospholipid membranes in theory and experiment***

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abstract:

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For the understanding of the interaction of nanoparticles (NPs) and living cells the interaction of NPs with lipid membranes is an integral step. During particle uptake, the membrane has to bend. The fact that, due to the nature of their phase diagram, the modulus of compression of these membranes can vary by more than one order of magnitude requires that both thermodynamic and mechanical aspects of the membrane have to be considered simultaneously. We demonstrate that silica NPs have at least two independent effects on the phase transition of phospholipid membranes: a chemical effect resulting from the finite instability of the NPs in water and secondly a mechanical effect which originates from a bending of the lipid membrane around the NPs.

We report on our recent experiments which allow to clearly distinguish between both effects and present a thermodynamic model including the elastic energy of the membranes, which correctly predicts our findings both quantitatively and qualitatively.

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Keywords: DSC, phase transition, supported lipid membranes, encapsulation, silicic acid

## I. Introduction

The increasing amount and variety of artificially produced nanometer scale particles calls for a thorough understanding of the influence of such nanoparticles on biological material. Especially the uptake in human cells and its consequences are in the focus of many research teams (1, 2). Silica nanoparticles (NPs) not only occur in exhaust emissions, but are also additives to food, textiles and construction materials to improve their properties (3). Moreover, they are even considered for drug delivery (4). In particular the uptake of NPs by living cells has recently become subject of risk assessments, as it correlates with cytotoxicity (5). In this context, it has been shown before that clathrin-dependent endocytosis is the most important pathway for the cellular uptake of silica-nanoparticles (6). For the detailed understanding of endocytotic mechanisms it is helpful to first study the mechanical properties of pure lipid membranes, which have been shown to be suitable model systems for cell membranes (7). Here, one has to bear in mind that the elastic properties of the lipid membrane depend on its thermodynamic state and can vary substantially. Especially during the transition from the gel to the fluid phase, the bending modulus changes by at least one order of magnitude (8, 9). Indeed, it has been demonstrated that this phase transition can trigger a variety of morphological transitions even in the absence of membrane proteins. Tube formation, fission, budding as well as the expulsion of entire vesicles have been reported (10-13). The thermodynamic state of the membrane must therefore be definitely considered as an important factor for the understanding of transport mechanisms in cells. The lipid phase transition can be conveniently monitored by Differential Scanning Calorimetry (DSC) which allows to detect even minor changes in the membrane properties (14). In 1992, Naumann et al. were able to demonstrate that 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) bilayers on a spherical particle support ( $R \approx 300\text{nm}$ ) melt cooperatively, but exhibit a suppressed pre-transition (15). Using particles of two different sizes ( $R \approx 30\text{nm}$ ,  $R \approx 300\text{nm}$ ), it has further been shown that the transition temperature of supported membranes is related to their curvature, i.e. the radius of the particles (16). Finally, for vesicles with diameters below 100nm (17), the same concept has been applied to analyze the influence of differences in the curvatures of the inner and outer leaflet on the melting transition temperature  $T_m$ .

Here, we employ DSC to characterize the impact of [the same NPs that have been shown to be cytotoxic for human endothelial cells](#) (5) on different phospholipid membranes. We investigate spherical supported vesicles (SSV) in terms of their transition temperature and find significantly different dependencies on the membrane curvature for different lipid-chain-lengths. A thermo-mechanical model is developed being able to explain our experimental findings by including the bending energy of the bilayer into the thermodynamic potential. In addition, a chemically induced depression of  $T_m$  is reported, which is triggered by the release of small amounts of silicic acid (SA) from the NPs.

## II. Materials and Methods

Silica particles with the following diameters were used:  $d = 16 \pm 2$  nm,  $18 \pm 2$  nm,  $85 \pm 4$  nm,  $212 \pm 25$  nm,  $305 \pm 35$  nm and  $348 \pm 40$  nm. All NPs used were synthesized and analyzed as described earlier in detail by Blechinger et al. (6). 1,2-Ditridecanoyl-*sn*-glycero-3-phosphocholine (13:0PC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC or 14:0PC), 1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine (15:0PC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC or 16:0PC) and 1,2-diarachidoyl-*sn*-glycero-3-phosphocholine (20:0PC), dissolved in chloroform, were obtained from Avanti polar lipids (USA) and used without further purification.

Suspensions of vesicles and NPs were prepared by re-hydrating the dried lipid film with a dispersion of NPs in ultrapure water (pure Aqua, Germany;  $18,2\text{M}\Omega\text{cm}$ ). The final lipid

concentration was 1 mg/ml. For the multi lamellar vesicle (MLV) preparation, the sample was heated above the main phase transition temperature  $T_m$  for 60 minutes and vortexed several times. Afterwards, the solution was either sonicated above  $T_m$  for 30 min or directly loaded into the calorimeter. The pure NP dispersions as well as the SSV containing sonicated samples were analysed regarding their colloidal stability. Dynamical light scattering analysis shows that the hydrodynamic radius of such samples does not change significantly over typical experimental timescales, i.e. several hours. Also the observation of different dried samples by SEM shows no signs of big agglomerates.

The measurements were carried out with a Microcal VPDS Differential Scanning Calorimeter (18) at a scan rate of 17 K/h. The reference sample was ultrapure water. The supernatant of centrifuged NP dispersions was analyzed with electrospray ionisation mass spectrometry (ESI-MS, Thermo Finnigan LTQ FT, resolution 100.000 at  $m/z=400$ , up to 2000u, 4kV, heating capillary temperature 250°C) to quantify the most frequent oligomers of silicic acid released from the NPs.

### III. Results and Discussion

#### Chemical impact of silica NPs on $T_m$

When adding NPs to a suspension of MLV, both the main transition and the pre-transition peaks shift towards lower temperatures (see Figure 1A). To quantify this effect, the peak position of the main transition ( $T_m$ ) was analyzed as a function of particle concentration and size (figure 1B). The mass concentration  $c_m$  was chosen such to keep the total NP surface area per sample in the same range for all NP sizes. Thus,  $c_m$  covers a wider range for the larger NPs (figure 1B). Our experiments revealed the following trend: independent from particle size, the temperature shift of the main transition exhibits a *linear dependence on the total amount of added NP mass* which indicates an effect that is *not* related to the total particle surface. The measurements were then repeated substituting the particles by the supernatants of the centrifuged NP dispersions of adequate particle concentration (10000g, 3min-300min, depending on NP size). Figure 1B shows that the temperature shift of such NP free samples is equal to the corresponding NP containing samples. Therefore, the shift in  $T_m$  with increasing concentration *does not* arise from the physical presence of the NPs, but can rather be caused by substances dissolved from the NPs. This is confirmed by the fact that an increase in incubation time of washed NPs, leads to an increasing shift in  $T_m$  as well. Alexander et. al. (19) already reported that silica always partially dissolves in aqueous solutions, forming silicic acid oligomers (SA). Using equation 42 of (20) for the used NPs, 95% of the equilibrium concentration of silica in water is reached within nine hours, leading to a concentration of SA in the range of 150 ppm (19, 21). ESI mass spectra showed both tetramers ( $m/z = 274.8809$ ) and pentamers ( $m/z = 370.8693$ ) are the most frequent oligomers in our NP dispersions (data not shown).

To confirm that SA is indeed the origin of the decrease in  $T_m$ , freshly prepared and oversaturated SA solution was added to MLV samples. A linear decrease in  $T_m$  as presented in figure 2 was found.

Regarding the hydrophilic character of SA (22) we expect head group effects to cause the observed melting point depression. One possible explanation could be an altered solvation of both lipid phases in a SA solution compared to water.

Disregarding the specific nature of the SA – lipid interaction, we assume that the soluted SA influences the chemical potentials of gel phase  $\mu_{gel}$  and fluid phase  $\mu_{fluid}$ :

$$\mu_i(T) = \mu_i^w(T) + \Delta\mu_i^{tr}(T) \quad (1)$$

$\mu_i^w$  denotes the standart chemical potential of the corresponding lipid phase in water and  $\mu_i^{tr}$  the free energy associated with a transfer of the phase from water to the solution.

It was shown earlier (23) that for the shift of the transition temperature  $\Delta T_m$  holds:

$$\Delta T_m = \left[ -\frac{RT_m^2}{\Delta H(T_m)} \right] \alpha c_{SA} =: A c_{SA} \quad (2)$$

where  $R$  is the gas constant,  $T_m$  the melting temperature of the lipid in pure water,  $\Delta H(T_m)$  the change of the systems enthalpy at  $T_m$  and  $c_{SA}$  the concentration of the solute SA. For low concentrations  $c_{SA}$  and small temperature changes,  $\alpha$  is a constant that depends on the strength of the interaction of the solute with both phases, i.e. the values of  $\mu_{gel}^{tr}(T_m)$  and  $\mu_{fluid}^{tr}(T_m)$ .

It is noteworthy that the linear dependence of  $\Delta T_m$  on  $c_{SA}$  is true for a direct associative reaction between lipid phases and solute as well as for indirect influences as solvation effects, for instance. Our measurements with oversaturated SA (see fig. 2) suggest a proportionality constant of  $A = 0,119 \text{ mK/ppm}$ .

A very interesting result is that the equilibrium concentration of (SA) in water seems to depend on the amount of dispersed nanoparticles, a phenomenon that cannot be explained when the particles are simply considered as small bulk material but a further examination of that point would be out of the scope of this work.

Here we want to emphasize that silica nanoparticles release amounts of SA which can significantly influence the thermodynamic properties of lipid membranes. The concentration of dissolved SA seems to be approximately proportional to the mass concentration of nano particles in the dispersion, and does not dependent on their size. Future studies should take that effect into account, regardless of the curvature-induced effect explained in the following section.

## Development of spherical solid-supported phospholipid bilayers

Storing the samples for one week after preparation led to the occurrence of an additional peak in the DSC profile at a temperature  $T_s$  (see Fig.3). After sonication, this additional peak becomes more pronounced than the original one at  $T_m$ , indicating an increasing portion of the lipids undergoing the transition at  $T_s$  following this step. Furthermore, the samples were centrifuged after sonication at 10000g for 15 to 60 minutes. Before repeating the centrifugation step, the supernatant was replaced with ultrapure water. In the inset of figure 3, the ratio of the transition enthalpies of the additional ( $\Delta H_s$ ) and the main transition ( $\Delta H$ ) are shown before and after centrifugation. While this ratio is only about 0.01 for the untreated sample, it increased to 1 after the first and to 2.2 after the second centrifugation step. This clearly indicates that the additional peak at  $T_s$  has its origin in lipids which are attached to the NPs.

This observation and conclusion is consistent with the findings of Naumann et al. and Bayerl et al. (15, 24) who coated NPs with lipid membranes and reported comparable shifts in  $T_m$ . Furthermore, in 1996, Brumm et al. showed some differences in the curvature dependence between 14:0PC and 18:0PC, but these reports left the question about a systematical study of the influence of the NP size open.

Hence, the heat capacity profile of the supported membrane population was analyzed both for NPs of different diameters (20 nm - 348 nm) and lipids with different chain lengths between 13 and 20 carbon atoms but identical head group. To account for the above mentioned chemical melting point depression, we analyzed the difference  $\Delta T := T_m - T_s$  (see figure 4A) between the transition temperatures of free and supported lipids. Assuming that the above *chemical* effect is of the same order for both experiments, it should cancel out.

All measurements show the same tendency, namely a shift of  $T_m$  towards lower temperatures for the solid-supported case. Exemplarily, for 20:0PC the heat capacity profiles are shown in figure 4A for different NP sizes. The expected broadening of the SSV due to decreasing cooperativity with increasing membrane curvature was observed but not analyzed further.

Figure 4B shows  $\Delta T$  for different lipids and NP diameters. While  $\Delta T$  decreases with decreasing NP diameter for 13:0PC, 14:0PC and 15:0PC, it increases for 16:0PC and 20:0PC. For all lipids, except for 20:0PC, the limit for a “flat” support of zero curvature is roughly  $\Delta T = 2,5$  K.

To summarize, our results consist of three main observations: i) for all lipids and NP sizes,  $T_s$  shifts towards lower temperatures, with a  $\Delta T$  ranging between 0.5 K and 4 K, ii) the curvature dependence changes its sign for increasing chain length and iii) for all lipids,  $\Delta T$  shows a saturation behavior with decreasing curvature.

### Theory. Bending contribution to $\Delta T$

In the case of SSV, the vesicles spread on and cover the NPs or parts of them, hence experiencing a curved substrate. In the following paragraph, an analytical expression for the expected change in phase transition temperature  $T_m$  based on the mechanical and calorimetric properties of the system will be derived. We therefore integrate the bending energy of the membrane in a Landau-type potential. No new or additional model assumptions are introduced. Instead, we combine existing theories to provide a coherent explanation of our results.

#### *Contributions to the Landau potential*

In the Landau theory, a first order phase transition is represented by the relative evolution of the double well potential of the form shown in figure 5 (25, 26):

$$\Phi(P, T, \Pi, \eta) = \Phi_0 + A\eta^2 + B\eta^4 + C\eta^6 \quad (3)$$

Here,  $\eta$  is the order parameter and  $A(T, p, \Pi)$ ,  $B(p, \Pi)$ ,  $C(p, \Pi)$  are functions of the thermodynamic variables temperature  $T$ , bulk pressure  $p$  and lateral pressure  $\Pi$ .

It is convenient and common to consider only the evolution of the potential minima in  $\eta$  with temperature. These are then identified with the Gibbs Free Energy potentials for the gel phase and the fluid phase (27, 28) as indicated in figure 6.

In the absence of any solid support, the two potentials intersect at the phase transition temperature  $T_m$ . The additional energies  $\Delta G_{gel}$  and  $\Delta G_{fluid}$  (see Fig. 5) in the presence of the support shifts the intersection towards lower temperatures  $T_s$ . For the transition temperature  $T_s$  now holds:

$$G_{gel}(T_s) + \Delta G_{gel} = G_{fluid}(T_s) + \Delta G_{fluid} \quad (4)$$

Assuming  $p$  to be constant and  $\frac{\partial^2 \pi}{\partial T^2} \approx 0$  as shown in (29),  $G_{gel}(T)$  and  $G_{fluid}(T)$  can be approximated by a first order Taylor series near the transition point  $T_m$ :

$$G_{gel}(T_m) + \frac{\partial G_{gel}}{\partial T} \Big|_{T_m} (T_s - T_m) + \Delta G_{gel} = G_{gel}(T_m) + \frac{\partial G_{fluid}}{\partial T} \Big|_{T_m} (T_s - T_m) + \Delta G_{fluid} \quad (5)$$

and consequently:

$$\Delta T := T_m - T_s = \frac{\Delta G_{gel} - \Delta G_{fluid}}{\left( \frac{\partial G_{gel}}{\partial T} \Big|_{T_m} - \frac{\partial G_{fluid}}{\partial T} \Big|_{T_m} \right)} =: \frac{\Delta G_{gel} - \Delta G_{fluid}}{\Delta \left( \frac{\partial G}{\partial T} \right)} \quad (6)$$

This general *analytical* expression connects the contribution of the solid support to the free energy  $\Delta G_{gel/fluid}$  with the shift of the transition temperature  $\Delta T$ .

$\Delta \frac{\partial G}{\partial T}$  *from the heat capacity profile*

$\Delta \frac{\partial G}{\partial T}$  can be extracted from the experimental DSC data, recalling the relation between the thermodynamic potential  $G$  and the heat capacity  $c_p$  as its susceptibility:

$$-T \frac{\partial^2 G}{\partial T^2} \Big|_p = T \frac{\partial S}{\partial T} \Big|_p = c_p \quad (7)$$

In agreement with the linear approximation that was described above, we have to integrate over  $\frac{\partial^2 G}{\partial T^2}$  (respectively  $c_p$ ) in the transition region to get  $\Delta \frac{\partial G}{\partial T}$ :

$$\Delta \frac{\partial G}{\partial T} = \int_{T_1}^{T_2} \frac{\partial^2 G}{\partial T^2} dT = - \int_{T_1}^{T_2} \frac{c_p}{T} dT \approx - \frac{\Delta H}{T_m} \quad (8)$$

The last approximation introduces the transition enthalpy  $\Delta H$  and holds for sharp transitions for which a constant temperature  $T_m$  can safely be assumed.

### ***Mechanical contributions to $\Delta G$***

In the case of SSV, we consider two main contributions to  $\Delta G$ : i) a curvature dependent one caused by the bending of the membrane and ii) a curvature independent one due to the bare presence of the substrate (planar limit). The latter, constant contribution to  $\Delta G$  can be caused by various interactions between lipids and support, for example by electrostatic forces.

To describe the mechanical contribution due to curvature, we use the well-known (30) expression for the bending energy  $E_{bend}$  of a membrane. For the curvature independent contribution we add an additional constant contribution  $G_s$ :

$$\Delta G = E_{bend} + G_s \approx \frac{1}{2} \left( \frac{2}{R} - \frac{1}{R_0} \right)^2 \kappa A_{mem} + \frac{1}{R^2} \kappa_G A_{mem} + G_s \quad (9).$$

Here,  $A_{mem}$  is the area of the lipid bilayer,  $\frac{1}{R_0}$  is the spontaneous curvature,  $\kappa$  the bending modulus and  $\kappa_G$  the modulus of Gaussian curvature. In the case of a chemically symmetric bilayer, the spontaneous curvature is caused by the asymmetry of the environment due to the NP and a thin layer of water inside and the bulk water outside the vesicle. The bending radius  $R$  is simply determined by the radius  $R$  of the particle.

Due to the higher flexibility of lipid membranes in the fluid phase, we assume a stronger contribution of the mechanical bending energy to  $\Phi$  for the low symmetric gel phase (fig. 5.). Therefore we neglect the bending energy contribution to  $\Delta G_{fluid}$  and hence the final expression for the temperature shift  $\Delta T$  can then be found by combining eq. 6, eq. 8 and eq. 9:

$$\Delta T = T_m \frac{\frac{1}{2}\left(\frac{2}{R}-\frac{1}{R_0}\right)^2 \kappa A_{mem} + \frac{1}{R^2} \kappa_G A_{mem} + G_{Sgel} - G_{Sfluid}}{\Delta H} \stackrel{\text{def}}{=} T_m \frac{\frac{1}{2}\left(\frac{2}{R}-\frac{1}{R_0}\right)^2 \kappa A_{mem} + \frac{1}{R^2} \kappa_G A_{mem} + \Delta G_s}{\Delta H} \quad (10)$$

This expression explicitly relates the shift  $\Delta T$  of the main phase transition temperature with the mechanical and the calorimetric properties of the membrane and predicts trends which can be compared to the experiments.

### ***Discussion – Bending energy explains the curvature dependence of $\Delta T$***

In figure 7, we show that our thermodynamic model can indeed well explain the observed trends of the temperature shift for different chain lengths. Eq. 10 was fitted to the data points of fig. 4B. Here  $T_m$  and  $\Delta H$  were taken from the heat capacity profiles,  $\kappa$  was set to  $2,5 \cdot 10^{-18} \text{J}$  for 16:0PC and estimated according to  $\kappa = 2,5 \cdot 10^{-18} \text{J}(h/16)^3$  for the other lipids (31, 32) where,  $h$  is the number of carbon atoms of the hydrophobic chains of the lipids. Furthermore  $A_{mem}$  was set as  $0,5 \text{nm}^2$  and  $R$  is the radius of the NPs. Thus there are three unknown parameters  $\Delta G_s$ ,  $\kappa_G$  and  $\frac{1}{R_0}$ . As the head groups of all lipids are the same it is reasonable to consider  $\Delta G_s$  as constant. For this reason we initially perform a three parameter fit to get an idea about the magnitude of  $\Delta G_s$  (see SM fig. 3a). Then  $\Delta G_s$  was set to a fixed value  $\Delta G_s = 150 \text{J/mol}$  resulting in a two parameter fit (fig. 7). Figure 7 shows an excellent qualitative and satisfying quantitative agreement between the analytical expression (eq. 10) and the experiments:

The order of magnitude, the range, the inversion of the curvature dependence and the quantitative shifts are predicted correctly. In the following the results for the parameters  $z := \frac{\kappa_G}{\kappa}$  and  $c_0 := \frac{1}{R_0}$  are discussed.

The fit results in values of  $z \approx -2$  (for details see SM fig. 2) in accordance with theoretical predictions (33, 34). In (33) it is shown that you can calculate  $\kappa_G$  as the second moment of the stress profile of the bilayer cross section. Using a simplified stress profile we show in the supplementary material that for lipids with the same head group  $\kappa_G$  results as a quadratic function of the chain length  $h$ , what is in good accordance with our values from the fit (see SM Fig.4). As also simulations of simple amphiphilic molecules show a similar dependence of the bending moduli as function of the chain length, the results seem convincing.

On the other hand the resulting values for the spontaneous curvature are more curious. As shown in SM fig. 2 for almost lipids we get negative  $c_0 \approx -0,005$ . For the exception 13:0PC the spontaneous curvature is almost negligible. Intuitively we would expect a decrease in spontaneous curvature with increasing membrane thickness. This is still an open question that we cannot explain yet. But at least as the origin of the asymmetry in our case lies in the environment and not in the membrane composition, it is not surprising that we extract smaller values than those reported earlier (35).

Further sources of error can be the omitting of the bending energy contribution of the fluid phase to  $\Delta G$  and the assumption of a constant  $A_{mem}$  for all lipids. But the aim of this work was not the precise determination of the exact values of the spontaneous curvature of this particular case of silica NP and those lipids. We looked for a coherent model that predicts the right order of magnitude of  $\Delta T$  and the occurring trends with increasing curvature. For sure we can reason that  $z \approx -2$ , as otherwise the resulting bending energy contributions would already predict for relative small NP curvatures much too big shifts in melting temperature.

## IV Conclusion

In summary, we present in-depth theoretical and experimental studies on nano particle – membrane interaction showing that silica NPs are able to influence the thermodynamic state of lipid membranes via at least two different mechanisms. First, a melting point depression caused by silicic acid released from the NPs in aqueous solution has been clearly identified. This finding calls for a thorough analysis of the chemical stability of NPs before studying their interaction with biological matter, because even minute amounts of membrane-soluble substances can change the thermodynamic properties of the membranes significantly. Apart from this rather chemical aspect, we also find indeed a size dependent impact of silica nanoparticles on the thermodynamic properties of phospholipid membranes mediated by the bending energy of the membrane. An analytical expression to describe the shift in  $T_s$  of solid-supported lipid bilayers by thermodynamic and mechanical considerations was proposed. Together with further experimental data this could offer a way to estimate the modulus of Gaussian curvature of lipid membranes, a parameter that is very difficult to access otherwise. Finally, we like to point out that earlier work has demonstrated, that ion permeability, morphological changes or adhesion phenomena can be controlled by a shift in the thermodynamic state of the lipid membranes (11, 12, 27, 36). A comparison between these data and the shift in state by NPs observed here, demonstrates that NPs are in principle capable to induce the phenomena mentioned. We believe that these induced changes in membrane state are of biological relevance and it will be highly interesting to test this hypothesis systematically in experiments similar like those reported in Bauer et. al.(5), where we showed with identical NPs that the decrease in viability of cells was direct proportional to the total provided NP surface area. From our results we expect a shift in membrane order upon contact with the NP and correlation between the change in membrane phase transition and cytotoxicity.

## Acknowledgement:

We thank our cooperating group of Prof. C. Bräuchle, Ludwig-Maximilians-Universität, Munich for the mass spectroscopy measurements. This work was financially supported by the Nanosystems Initiative Munich and by the DFG through the SPP1313 BIOMEM (A. Reller, M. F. Schneider, S. Schneider, A. Wixforth).

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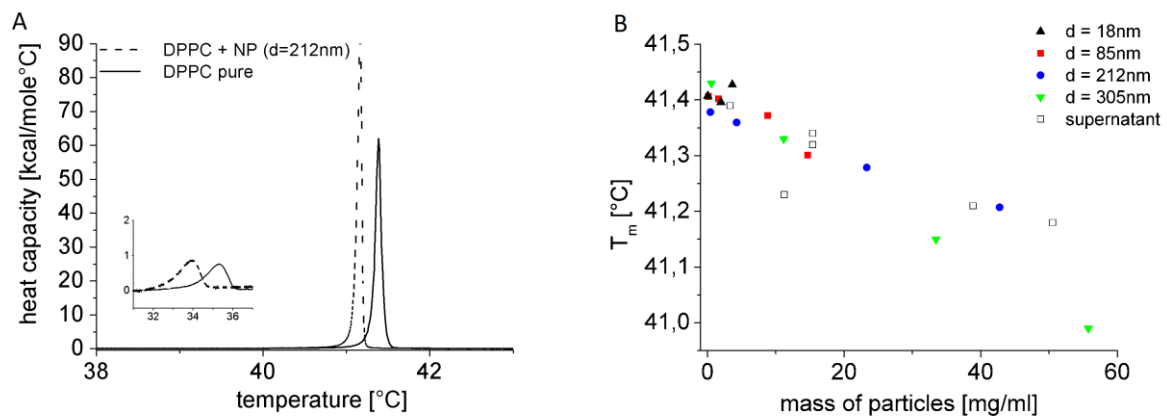


Figure 1

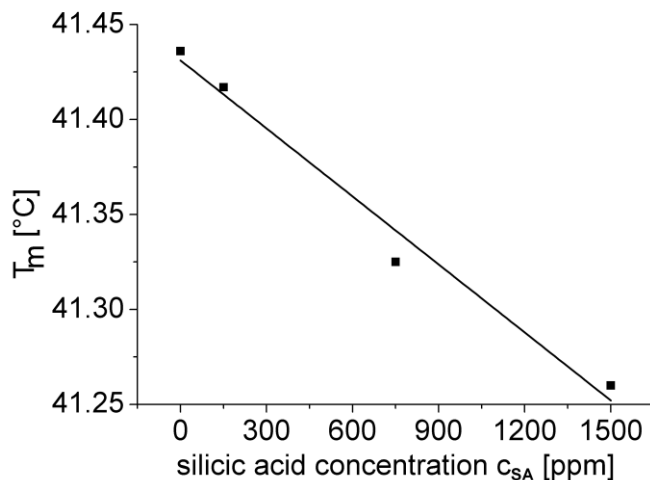


Figure 2

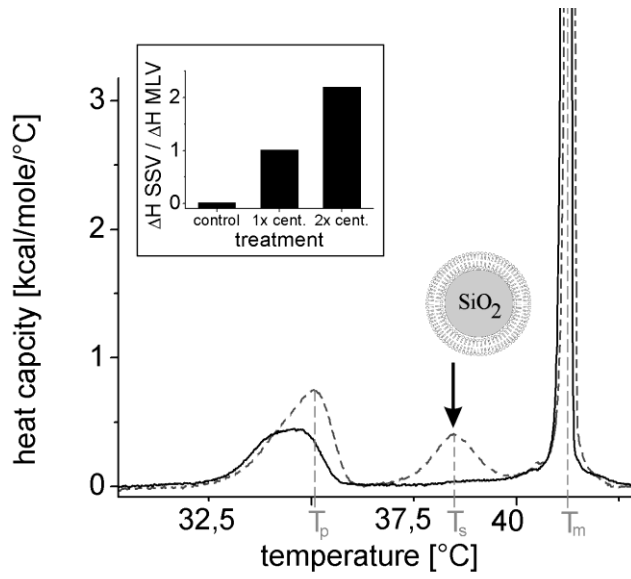


Figure 3

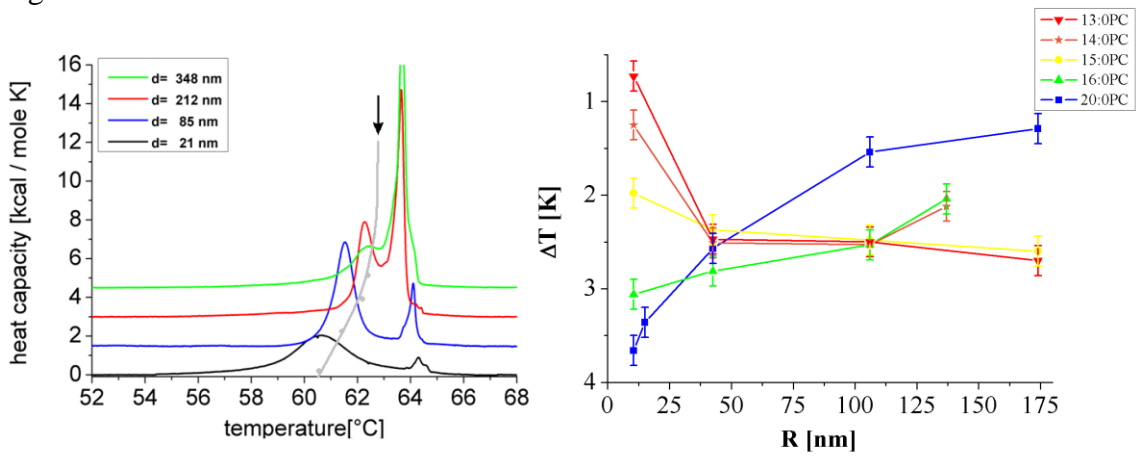


Figure 4

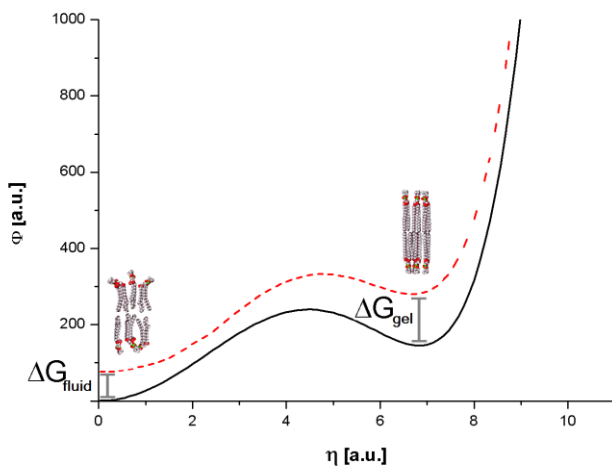


Figure 5

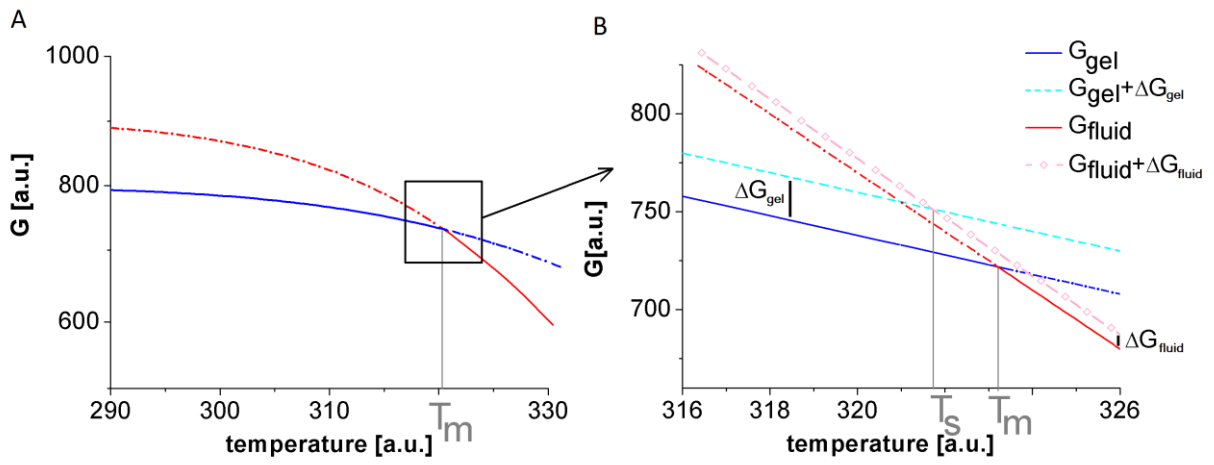


Figure 6

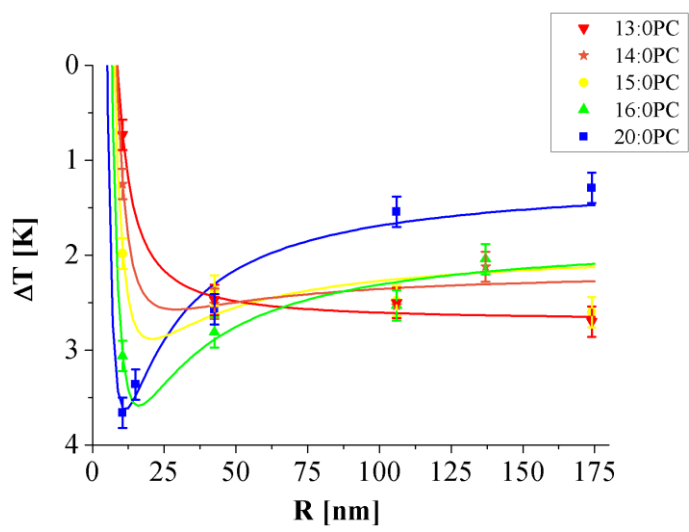


Figure 7