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A MICROTHERMODYNAMIC INTERPRETATION OF FLUID STATES FROM FTIR MEASURES IN LIPID MEMBRANES.

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ABSTRACT

Fourier Transform Infrared spectroscopy (FTIR) is usually employed to obtain transition temperatures of lipids and lipid mixtures and the effect on it of several effectors, such as cholesterol. However, no interpretation of the molecular information provided by the frequency shift to higher values observed at T_c is available. In this paper, we demonstrate that data obtained by means of FTIR measurements contain information about the microscopic thermodynamics of the lipid phase transition. By means of Monte Carlo simulation, we have been able to show that the frequency shift from low to high values can be taken as a two state transition of molecular constituents in a lattice rearrangement. According to the model, at temperatures below T_c all the groups are defined in the lowest energy state defined by the lowest frequency value and therefore they are all connected in a gel lattice. Above T_c, some groups may reach different energy states depending on the restrictions imposed to the groups. Ideally, when all the groups are able to reach the highest frequency, a fully "fluid" state is reached, meant as a disordered state. Taken this hypothetical state as reference it is possible to show that the higher states become less accessible. The model is suitable to describe the effect of cholesterol, which is able to dump the phase transition, and is congruent with previous data denoting that in the so-called fluid phase the first 4-5 methylene groups remain in the gel state even above T_c. The frequency value attained above T_c depends on the nature of the lipid acyl chain.

Keywords: methylene groups, FTIR, lipid membranes, asymmetric frequency shift, Monte Carlo,

INTRODUCTION

Gel to liquid crystalline phase transition of phospholipid bilayers has been studied by different experimental methods. The thermodynamic features of the phase transition have been obtained by differential scanning calorimetry, which provides the transition temperature (T_c), the enthalpic change at the phase transition and details of the onset of the phase transition [1-4].

On the other hand, studies with deuterium and proton nuclear magnetic resonance, fluorescent and electron paramagnetic (EPR) spectroscopies have indicated that membrane undergoes an order-disorder transition at T_c [5-9]. The phase below the transition temperature is denoted as a solid crystalline state and above it as a liquid crystalline phase. This phase is usually identified, in a misleading way, as "fluid", reflecting with this term an unorganized state as in a liquid[10].

As EPR and fluorescent spectroscopies use probes located at different depth of the bilayer, it has been possible to demonstrate that disorder in the lipid acyl chains propagates into the bilayer interior, being the first 4-5 methylene groups of the acyl chain unperturbed at the phase transition[5, 11]. Both, the main transition temperature and the enthalpic change strongly depend on the unsaturation, ramification and length of the acyl chains and on the head group composition[12, 13]. In addition, the phase transition enthalpy is severely decreased by the inclusion of cholesterol[12, 14, 15].

In parallel, it has also been reported that the amount of water associated with the lipids increases drastically when the transition occurs. Below T_c, the average hydration number per phosphatidylcholine is around 7 water molecules per lipid, which increases to 18-22 above the phase transition[16, 17]. Taken together, the increase in disorder in the methylene groups beyond the first four C-atoms is concomitant with the increase in the number of water molecules per lipid. This is one of the reasons by which the decrease in refractive index and turbidity of lipid dispersions has been explained. The decrease in refractive index is directly related with the density of the material which is changed by the entrance of water into the lipid matrix at T_c[18]. Schematically, the thermal agitation of the acyl chains due to heat absorption allow the formation of spaces that can be occupied by water molecules which in turn favors the transition. The concourse of heat and water penetration makes the transition temperature of fully hydrated lipids to be 80^{o}C below the melting point of the anhydrous lipid state [19,20].

Another method that has been employed to determine the phase transition is Fourier Transform Infrared Spectroscopy (FTIR) [21-23]. Several peaks assigned to the vibrational modes of different groups of the phospholipid molecule can be followed at different temperatures which provides a tool to measure nanoscale domain size and chain order parameters[15, 22]. Among those, the symmetric or asymmetric stretching of the methylene groups (CH_2) in the acyl chains is one of the most frequently used variables. A typical curve is one in which the frequency variation of the asymmetric stretching wavenumber of the C-H of different phospholipids goes from a 2850 to 2852 cm^{-1} as a function of temperature, with an inflexion point at T_c.

This type of determinations has been frequently used operationally but no further analysis is available. Spectral changes at the phase transition have been assigned to changes in the fractions of two well-defined states (gel and fluid) of the whole molecule[24]. However, as FTIR spectroscopy provides a microscopic view of the transition process focusing on chemical groups of the lipid molecule without the inclusion of probes this kind of measures can be interpreted in terms of intermolecular interactions. The plot of the of the asymmetric stretching mode wavelength of the CH_2 groups as a function of the reduced temperature for a series of lipids gives a sigmoid curve centered at the phase transition temperature[25]. The frequency of the stretching mode is directly related to the constant force of the CH bonds in the phospholipid. This constant force may change due to lateral interactions of the bond with the adjacent groups, either of the same specie or others. Hence, any variation in frequency indicates a change in the intermolecular interactions that make weaker the C-H bond. The low frequency population observed in the gel state corresponds to the stretching of the CH_2 residues having lateral contacts with similar adjacent groups of other lipid chains. This population is denoted as connected groups[25]. Above the phase transition the frequency increases, denoting stronger CH bonds, due to weaker lateral interactions. This population was denoted as isolated CH_2 populations. Figure 1 describes schematically the CH_2 groups and its interactions. This picture shows the organization of the acyl chains attached to the polar head groups in a bi dimensional lattice. This diagram will be the base of the model proposed below.

According to this approach, the CH_2 frequency shifts with the reduced temperature from connected to isolated CH_2 populations. In the gel state, same frequencies are obtained for connected populations for different types of lipids. However, the maximum frequency attained above T_c is not similar for lipids with different acyl chains. The frequency is higher in unsaturated or branched lipids with respect to saturated ones and when the acyl chain length increases. That is, although above the transition all lipids reach an unorganized state

(operationally identified as "fluid" states), they differ in the entropic state at microscopic level as inferred previously from DSC measurements[10]. This may be due to the fact that with the increasing acyl chains the four first CH_2 remains unable to follow the transition independently of the acyl chain increase. This region is shown in Figure 1 as that confined between dotted lines.

That is, the so-called fluid state does not correspond to a complete disorder in the membrane phase. In addition, considering the changes in hydration also occurring at the phase transition, the increase of isolated CH_2 population above T_c is concomitant with the occupation of holes by water molecules within the lipid lattice[23, 26].

Computer simulation in the last years has given a new vision of the thermodynamics of the membranes. All the Monte Carlo techniques applied to critical phenomena can be used in biology issues[27-29]. In all these cases, the lipid membrane has been considered as a two state-like Ising model. The gel–fluid phase transition has been tackled with this technique[27, 28]. Those previous molecular simulations consider up to 300 x 300 lattices in which each node is represented by a lipid acyl chain[30].

This is a macroscopic interpretation of the melting process based on calorimetric data but gives no details on the molecular "state" of the lipid components (internal modes). In these approaches no details about the state of the CH_2 groups at different depth is considered. Moreover, the fluid state is taken as if all the components in the whole molecule (acyl chain and head group) go to a disordered state as the melting of the bidimensional lattice. However, this view is not congruent with the order parameters measured by EPR, in the sense that the first four methylene groups do not melt. In consequence, this approach cannot report the different states of the lipids at temperatures above the critical transition temperature as observed experimentally.

The purpose of this work is to interpret the information derived from FTIR measures under the frame of a molecular simulation model to obtain new understanding of the behavior of the different states reached above the phase transition temperature according to the lipid acyl chain nature and the addition of cholesterol.

METHODOLOGIES.

Lattice- gas model

A membrane can be visualized as a two-state model. The gel-to-liquid crystalline transition is described as a lattice gas model. The lattice gas model is applied to space between both polar

head groups, as shown in Figure 1. In this figure, the black dots are the polar heads and the blue dots are the apolar groups (CH_2). In a DPPC membrane the hydrocarbon chains form a triangular lattice (shown with dash green lines in Figure 1). In a triangular lattice each lipid can be in two states: fluid (F) or Gel (G). The coordination of each lipid is six.

For a membrane of size L, and M=L x L sites (lipids unit), here N=L/2 is the length of a chain[24, 30]. The transition of each unit are characterized by a change in the Gibbs energy between the trial and original conformation as:

$$\Delta G = \pm(\Delta H - T\Delta S) + \Delta n\omega \qquad (1)$$

where ΔH and ΔS are the changes in the enthalpy and entropy, respectively. *T* is the temperature in Kelvin's units, Δn is the change in the number of unlike nearest neighbors for a selected lipid when changing its state.

The fundamental change that is introduced with respect to previous models is that CH_2 groups are located in the sites of the lattice and are identified as nodes**.** A lipid unit is composed by several of these groups. Each group in this lattice cell can access to two states: one of low energy and one of high energy, characterized by the frequency of stretching according to

$$E = h\nu = \frac{1}{2\pi}\sqrt{\frac{k}{\mu}} \qquad [2]$$

where *k* is the constant force of the CH bond in the methylene groups and μ the reduced mass of C-H atoms.

In order to model the phase changes, according to the microscopic state given by molecular spectroscopy, a lattice gas model is considered, where each node of the lattice is a methylene group of the lipid unit having a defined position. The energy state of each group is given by the vibrational state of the bonds in the CH_2 group, according to equation 2. One frequency value describes molecularly the gel state for all lipid elements. Above T_c fluid elements can be described by a higher frequency value. Thus, the node can be defined in the gel state or in the fluid state according to its degree of freedom detected by the frequency of the stretching. This is justified because we measure with FTIR directly the state of the node by means of the frequency value. The connection of the groups in each element is constant at all temperatures. The transition occurs when the group in the element gets a given energy by heating.

The plus sign is used for a change from gel to fluid and the minus in the inverse sense. ω is the cooperativity parameter. This parameter takes into account the lateral interactions. With this hypothesis, all the others considerations are valid.

In order to proceed, we define a θ-parameter as the probability of a node (a CH_2 group in each lipid unit) to be unaltered during the transition. If θ=0, all the groups in the lipid unit are able to reach a high frequency state and in this state the membrane can reach the highest fluid state, i. e. all groups can reach this maximum state of entropy. This case would correspond to the typical approach found in literature [24, 30-35].

If θ=1 no group in the lipid unit is able to increase the disorder and the membrane is in the gel state. In this case, no transition is feasible. However, for θ between 0 and 0.5, only a fraction of the groups can reach the fluid state i. e. the high frequency state. In other words, a fraction of groups do not gain all the possible entropy increase.

Monte Carlo Simulations

We consider the membrane in the equilibrium state. To obtain the equilibrium in the system we use the Metropolis algorithm[36, 37] and the Gluber dynamics or single spin flip. In this algorithm, we choose randomly a node and we try to change its state. The probability of transition is computed using equation (1).

The dynamics for T is summarized as:

i) Initially the lattice is in the gel state and for a given value of θ.

ii) A node is chosen randomly and its change of state is done, comparing the energy given in equation (1) with a random number between [0, 1).

iii) Repeat ii) M times. This procedure is called a Monte Carlo Step (MCS).

iv) After the discard $1x10^5$ MCS, the quantities of interest are obtained by averaging over $1x10^5$ successive configurations separated from each other $1x10^5$ MCS.

The definition of a phenomenological order parameter (ρ) that gives information of the density of states at the membrane is important to describe the process:

$$\rho = \frac{1}{M}\sum_{i}^{M} s_i \qquad [3]$$

s_i is a local state variable of the node "i" and can take the values 1 or -1. If s_i =1(-1) the state for the node is L(G). If ρ =1 (ρ =-1) the membrane is in fluid phase (gel phase).

Thermodynamic quantities, such as enthalpy, H, heat capacity, C_p and order parameter are obtained by simple averaging. The heat capacity C_p, according to the fluctuation theorem can be written:

$$c_P = \frac{\langle H^2 \rangle - \langle H \rangle^2}{RT^2} \qquad [4]$$

where <…> denotes the time average over a given number of MCS at the equilibrium.

RESULTS

Test of the model.

Figure 2 shows the order parameter calculated according to equation 3, versus the reduced Temperature for L=32 (1024 sites) for the indicated different values of θ. In our model boundary periodic conditions is considered only in a direction perpendicular to the polar heads. The values of enthalpy ΔH=36.4kJ/mol, entropy ΔS=116 J K/mol and ω = 50 J/mol were fixed for typical values of DPPC membranes. With these values the critical point is around T_c=ΔH/ΔS=313.79. We observe a smooth behavior (like a sigmoid) of the curves which is similar to that obtained from FTIR measures[25].

For θ tending to 0, all groups in the lipids have the probability to gain energy and thus the lipid goes to a disordered state in the whole extension, i.e. all groups are able to gain entropy to increase intrinsic disorder. It is possible to obtain T_c from the inflexion point. At θ=0.0 we obtain T_c (θ=0.0) ≈313.5 K in concordance with the experimental data. The order parameter shows: full gel phase (ρ=-1) at T/T_C < 0.98 and a "fluid" phase at T/T_C> 1.04 (ρ=1). For intermediate temperatures there is a combination of both phases.

For all θ, the order parameter has different values at different temperatures and reaches a saturation regime for high temperatures. The saturation can be characterized by $\boldsymbol{\rho_s}(\theta)$. This regime implies that at high temperatures the membrane still has a density of gel like nodes immersed in a fluid environment even for very high temperatures. This means that the fluid state (defined by the higher frequency value for each lipid) is not accessible for all CH_2 groups in the acyl chain. The exception is when θ = 0, in which case ρ_s = 1. The inflexion point of ρ is

useful to estimate T_c *(L).* For this, in the insert of the Figure 2, the derivative of ρ versus *T* for L=32 is shown. The maximum of this curve can be associated to the critical point.

Taking the plots at reduced temperature, θ values higher than zero reflects that some groups remain in the gel state even above T_c. The value of θ is related with the total CH_2 groups in the acyl chains and with those of them that can suffer the transition to a higher state of energy (higher frequency). The saturation ρ_s of the order parameter plotted versus θ gives a lineal dependence as shown in the Figure 3. This Figure is equivalent to the observation that the higher frequency value decreases with the increase in saturation or the decrease in chain length[25]. Thus, the decrease in the ρ_s value with θ is congruent with the decrease in frequency for shorter chain length or lower unsaturation, for instance in going from C-18 acyl chain to C-14 acyl chain as observed experimentally[25].

Comparison of the model with experimental results.

There are two ways by which it is possible to modify the saturation value of ρ_s. One is that discussed above in which for a given number of nodes (CH_2 groups in a cell lattice) the number of nodes that cannot be modified in the transition increases, i.e. θ increases. The other one is that in which for a given number of frozen CH_2 unable to melt, θ value is changed by the increase of total number of nodes.

In this session, this model is applied to the interpretation of the thermodynamic transition process of two experimental cases: the phase transition of DPPC membranes with increasing ratios of cholesterol and the phase transition of PCs with increasing saturated acyl chain from C-14 to C-20.

When $\theta > 0$, the cooperativity decreases and the area of the curve of the derivative of the order parameter decreases. The shift produced in the temperature is nearly negligible. This is illustrated in Figure 4 for the order parameter ρ vs T for different values of θ, and could be correlated with the cases of phosphatidylcholines mixed with cholesterol up to a 40%[38]. The insert shows the corresponding derivative of curves ρ vs T. This description is congruent with the experimental observation that the saturation values of the order parameter decrease with cholesterol. The model predicts that the frequency values at temperatures below T_c are similar for different cholesterol content, which would be in agreement with the experimental values within the experimental error.

This would correspond for a case in which the total number of nodes is fixed and the number of nodes not susceptible to change increases. This resembles the case of a lipid of a given chain length (for instance 16 CH_2 groups) to which cholesterol is added. In this case, the maximum of the derivative decreases without significant change in T_c. We observe that maximum decreases as θ increases, i.e. when the number of groups that are able to reach the fluid state decreases. This means that the cooperativity decreases with the increase of methylene groups in the connected state.

The other case in which θ may have different values between 0 and 1 is when at constant number of frozen nodes, the total number of nodes increases with the increase in the acyl chain length. That is, for a number of 4-5 fixed CH_2, the total number can grow from 14, 16, 18 and 20 given as a result 10, 12, 14 and 16 nodes that may suffer the transition. In these cases, the saturation value increases with the acyl chain (Figure 5) and T_c displaces significantly with the acyl chain length in the right direction as observed experimentally. The values settled to each chain are expressed in the Table I. The insert A shows C_p versus T for each values of N. The maximum is shift to high temperature as N increase. The enthalpy of the melting curve can be determined from the integration of C_p. The insert B shows the values calculated from the simulations. Lineal dependence of the enthalpy increase with the chain length (N) is observed. In the insert C) the critical temperature is estimated versus N both results are in concordance with the results at the literature[12, 39].

DISCUSSION.

Previous simulation approaches have predicted the transition temperature of lipid membranes considering the transition of the whole lipid molecules from the gel to a fluid state without considering the internal modes of the each residue in the lipid molecule. This kind of models neglects the experimental fact that the first four–five methylene groups near to the head group does not contribute to the phase transition[5, 11] and remains in the gel state above T_c.

In the present analysis, we have defined a θ parameter that accounts for the ratio between the internal modes of the lipid molecules (identified as the CH_2 groups) that cannot suffer a transition with respect to the total. The consequence of considering some CH_2 groups that cannot suffer the phase transition is that the final state in the liquid crystalline state (the so called fluid phase) is dependent on the lipid specie and on the addition of a second component such as cholesterol.

The saturation value (ρ_s) in the liquid state decreases with: 1.- the increase in θ for a constant number of nodes, with negligible displacement of T_c (cholesterol case). 2.- the increase in total number of nodes maintaining a fixed number of 4-5 frozen nodes with a significant displacement of T_c.

A similar behavior between our model and the values of stretching frequency obtained by FTIR spectroscopy has been found. In Figure 6, the behavior predicted with this approach is compared with FTIR frequency values for DMPC and DPPC. It is observed that the model fairly reproduces the decrease for high values of ρ_s when the acyl chain length decreases from DPPC to DMPC.

It is the first time, to our knowledge that thermodynamics of the phase transition of lipids membranes is modeled from spectroscopic data. Previously, molecular simulation was applied to macroscopic thermodynamic data such as T_c, ΔH and ΔS.

In our case, the interpretation of the CH bonds in the CH_2 groups of the acyl chain as independent oscillators allow to define the gel and the fluid states in terms of the internal modes of the lipid molecules. There is a lowest value at temperatures below T_c that is common for different lipid species (i.e. different acyl chains). This corresponds to the macroscopically defined gel state. According to the relative number of total nodes (i. e. number of CH_2 in the acyl chains) to those that may not have access to a higher energy, the values of frequency changes at temperatures above T_c. This value may change due to the fact that the relative number of oscillators (nodes) decreases by the steric hindrance imposed by molecules interposed between the acyl chains such as cholesterol (with the consequence of reducing the enthalpy at the phase transition with a negligible shift in T_c) or maintaining a fixed number of 4-5 frozen nodes when the acyl chain length increases (i. e. the number of CH_2 that may reach the higher frequency increases and T_c shifts to higher values)

In conclusion, we have reproduced the phase behavior of lipid membranes of different acyl chain length and the effect of cholesterol from measures of the molecular state of the methylene residues of the acyl chains. Several interesting conclusion can be derived. One of them is that in the gel state all methylenes are energetically equivalent. In contrast, only a fraction of them can access to the higher energy state. Molecules such as cholesterol would promote the hindrance to access high-energy states. The increase in chain length permits to have more nodes to access to the high state.

Finally, with this approach it is possible to define a similar state of different lipid membrane composition. For instance, a given ρ_s can be reached with a pure lipid of a given chain length or for a lipid with a higher length when mixed with cholesterol. This means that there are several ways to fix a membrane state, which might determine the thermodynamic response of the membrane to the insertion of peptides, enzymes or permeants. Further studies in this regard are being carried out.

TABLE I

CALCULATED VALUES OF ENTHALPY ENTROPY AND T_C FOR LIPIDS OF DIFFERENT LENGTH (N)

N	ΔH [J/mol]	ΔS[J/mol K]	Tc[K]=ΔH/ΔS
14	27000	91.5	295.08
16	36400	116	313.79
18	45500	139	327.33
20	50000	147	339.2

LEGENDS TO THE FIGURES.

FIGURE 1

Schematic description of the model membrane denoting the contact between the CH_2 units of the same lipid molecule or of adjacent ones. Green lines denote the triangular lattice.

Dotted lines frame the first four-five CH_2 groups that do not suffer the phase transition.

FIGURE 2.

Order parameter (ρ) versus reduced temperature for several values of θ. Insert: derivative of ρ versus reduced temperature.

FIGURE 3.

Saturation values of ρ (ρ_s) versus θ taken from Figure 2.

FIGURE 4

ρ vs Temperature for a case in which the total number of nodes is fixed and the number of nodes not susceptible to change increases (cholesterol-like case). Insert: Behavior of the corresponding derivative.

FIGURE 5

Calculated curves for a case in which θ may have different values between 0 and 1 when at constant number of frozen nodes, the total number of nodes increases (increase in the chain length case). Insert: Variation of T_c and ΔH with θ.

FIGURE 6.

Comparison of the model with experimental data for DMPC (blue) and DPPC (orange).

Continuous lines represents the variation of ρ vs T according to the model and the dots corresponds to experimental values of wavelengths for each lipid from Ref. 25.

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Figure 1

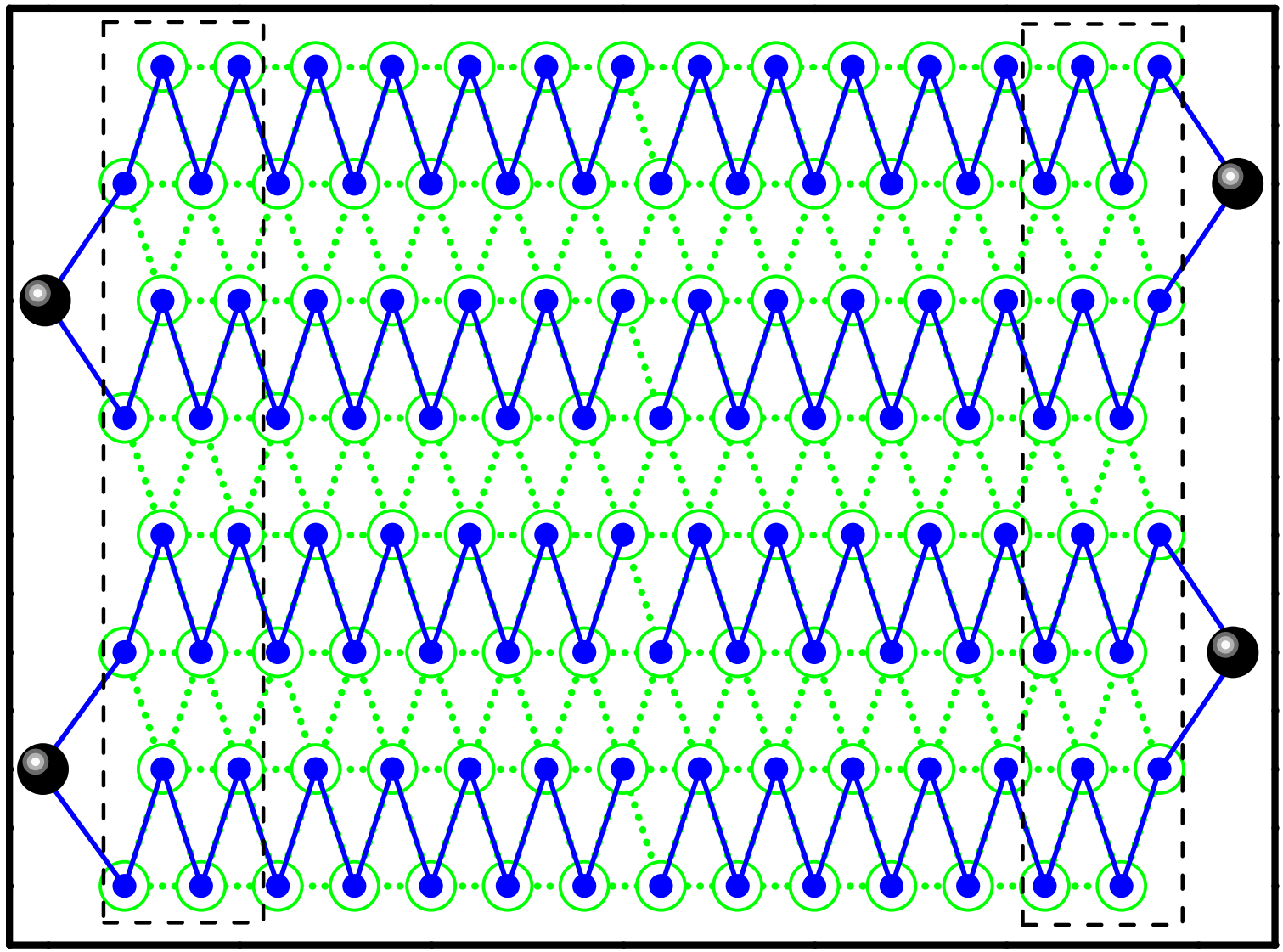
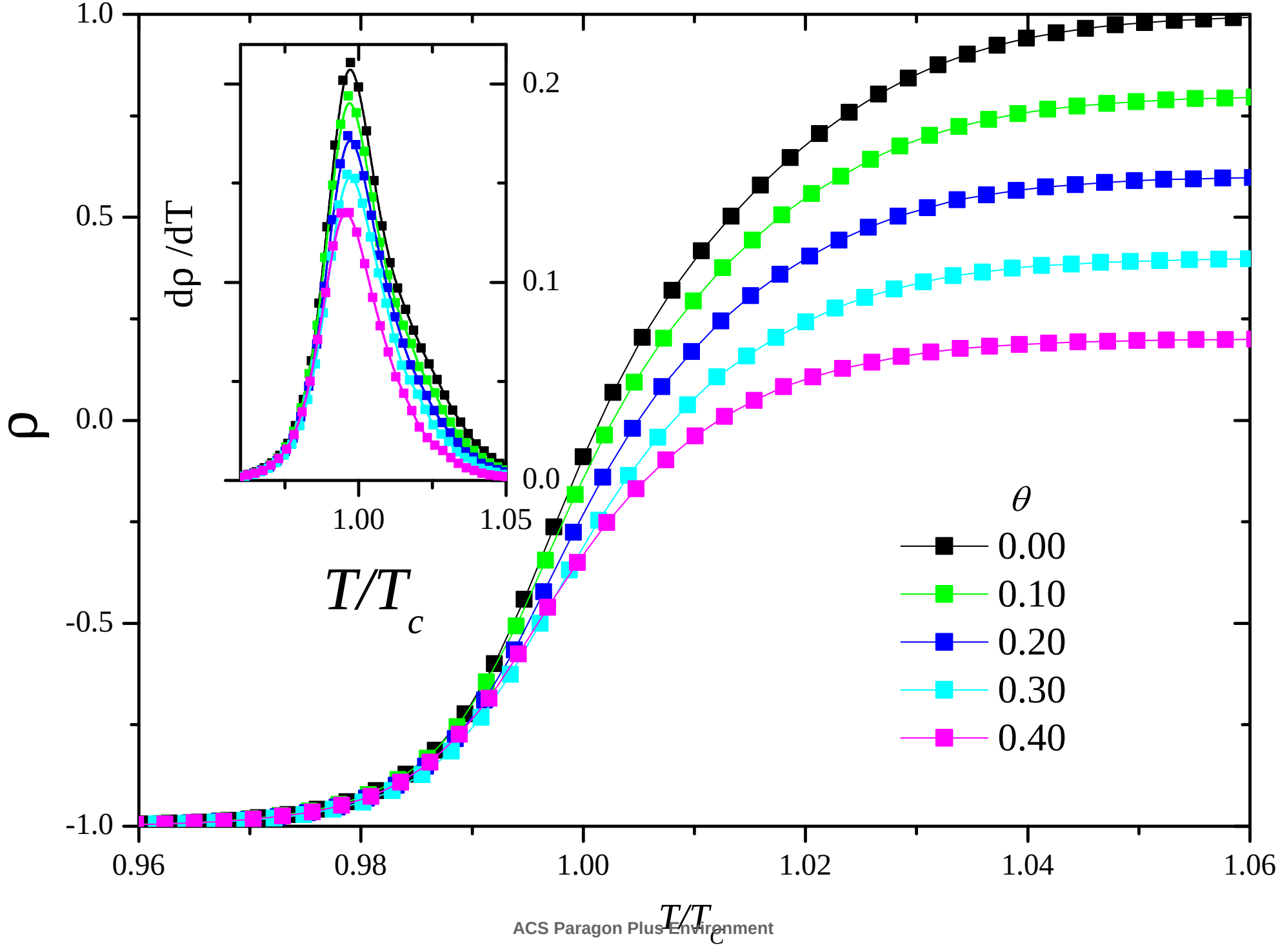


Figure 2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-1.0
ρ
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0.98
1.00
1.02
1.04
1.06
T/T_c
dρ /dT
0.2
0.1
0.0
1.00
1.05
T/T_c
θ
0.00
0.10
0.20
0.30
0.40

Figure 3

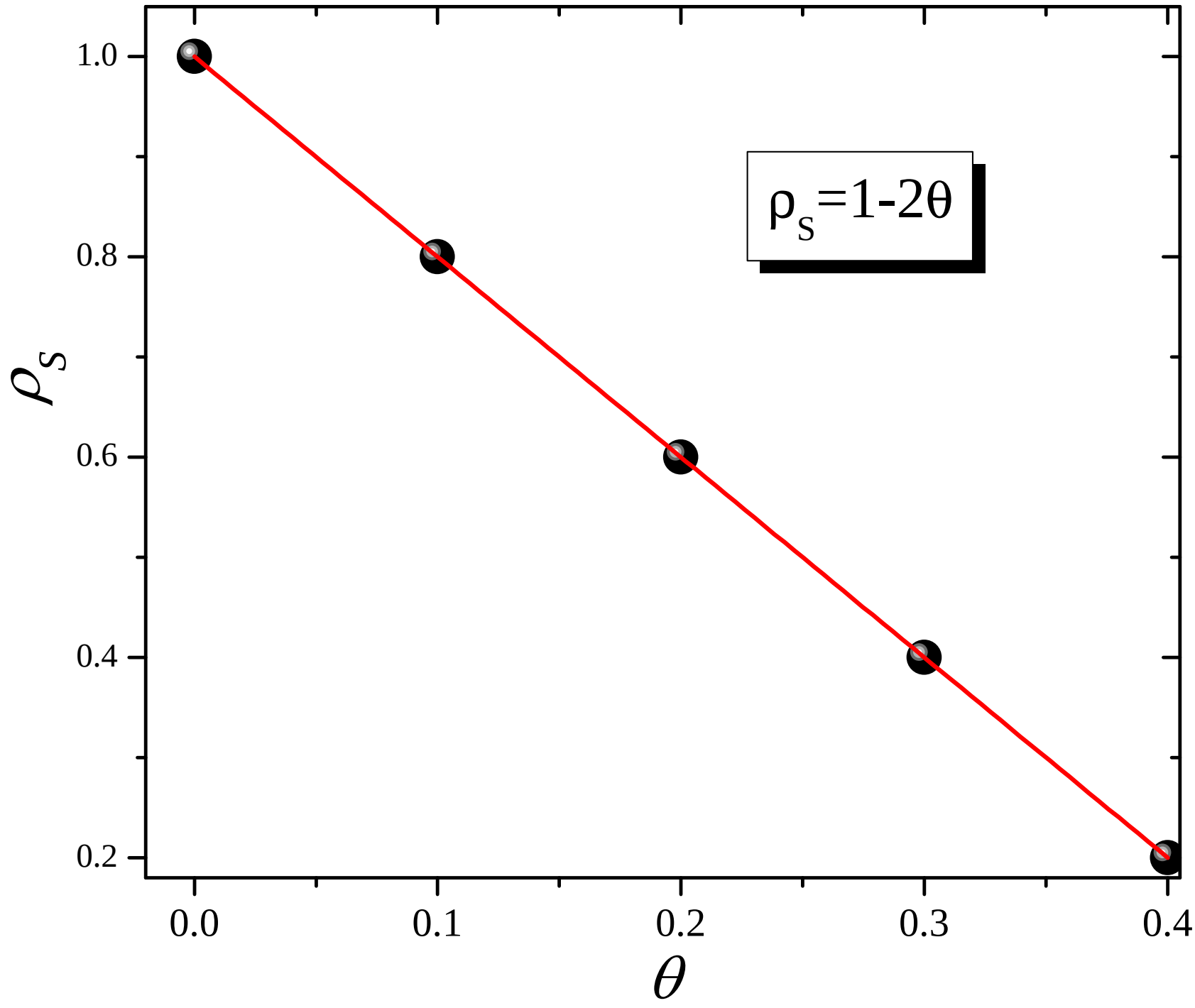
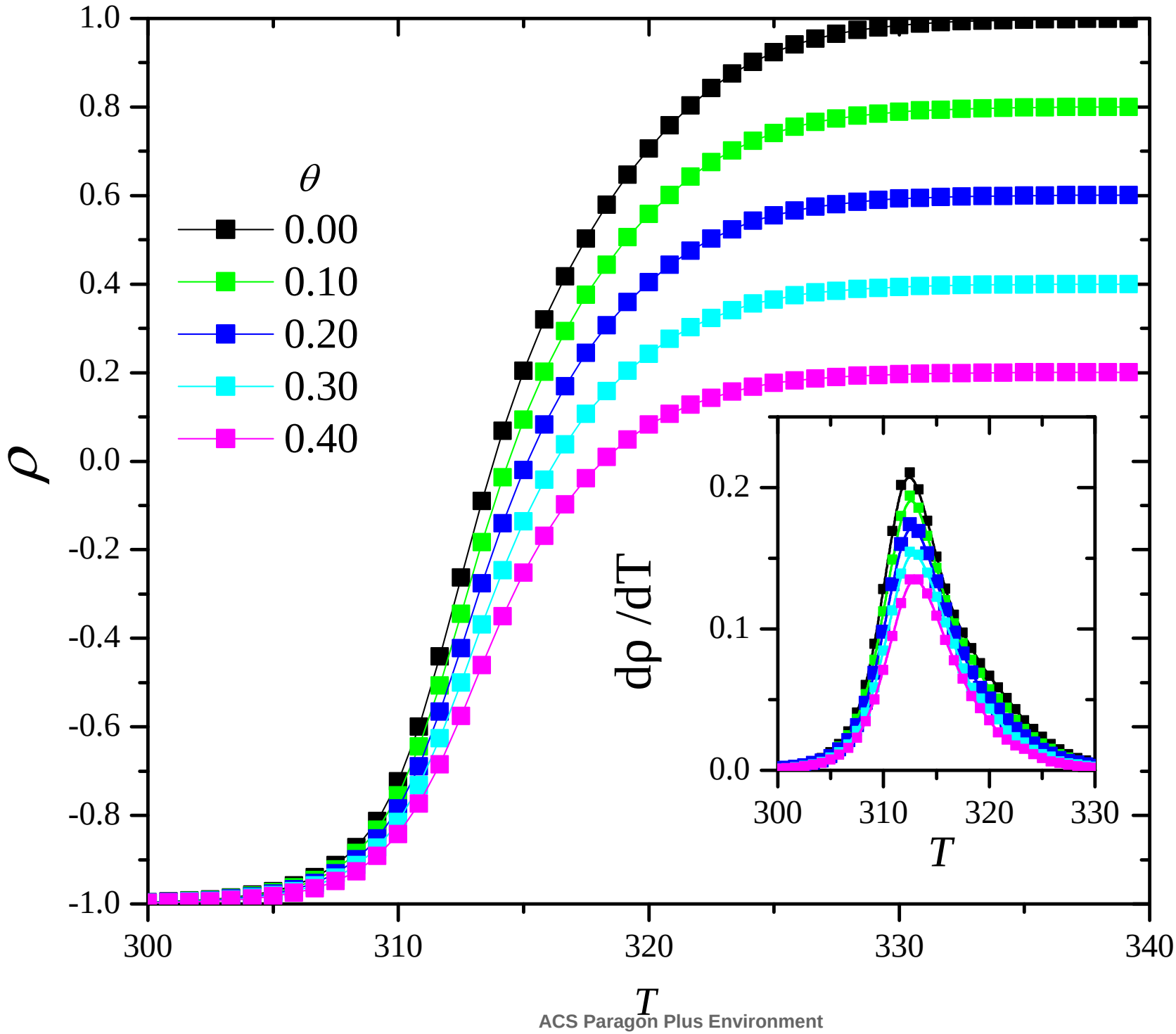


Figure 4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0.2
0.0
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-0.4
-0.6
-0.8
-1.0
ρ
θ
0.00
0.10
0.20
0.30
0.40
300
310
320
330
340
T
dρ /dT
0.2
0.1
0.0
300
310
320
330
T

Figure 5

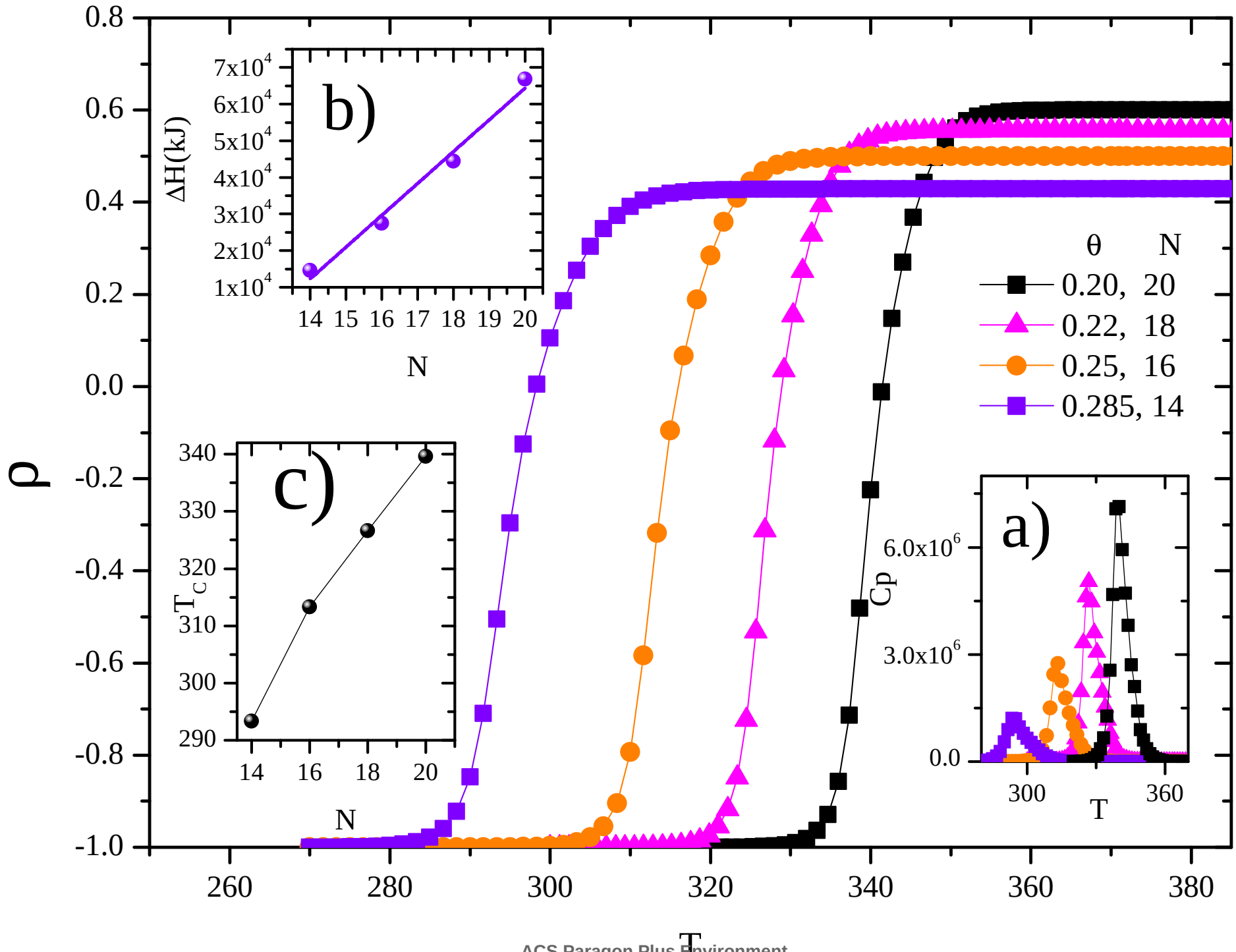
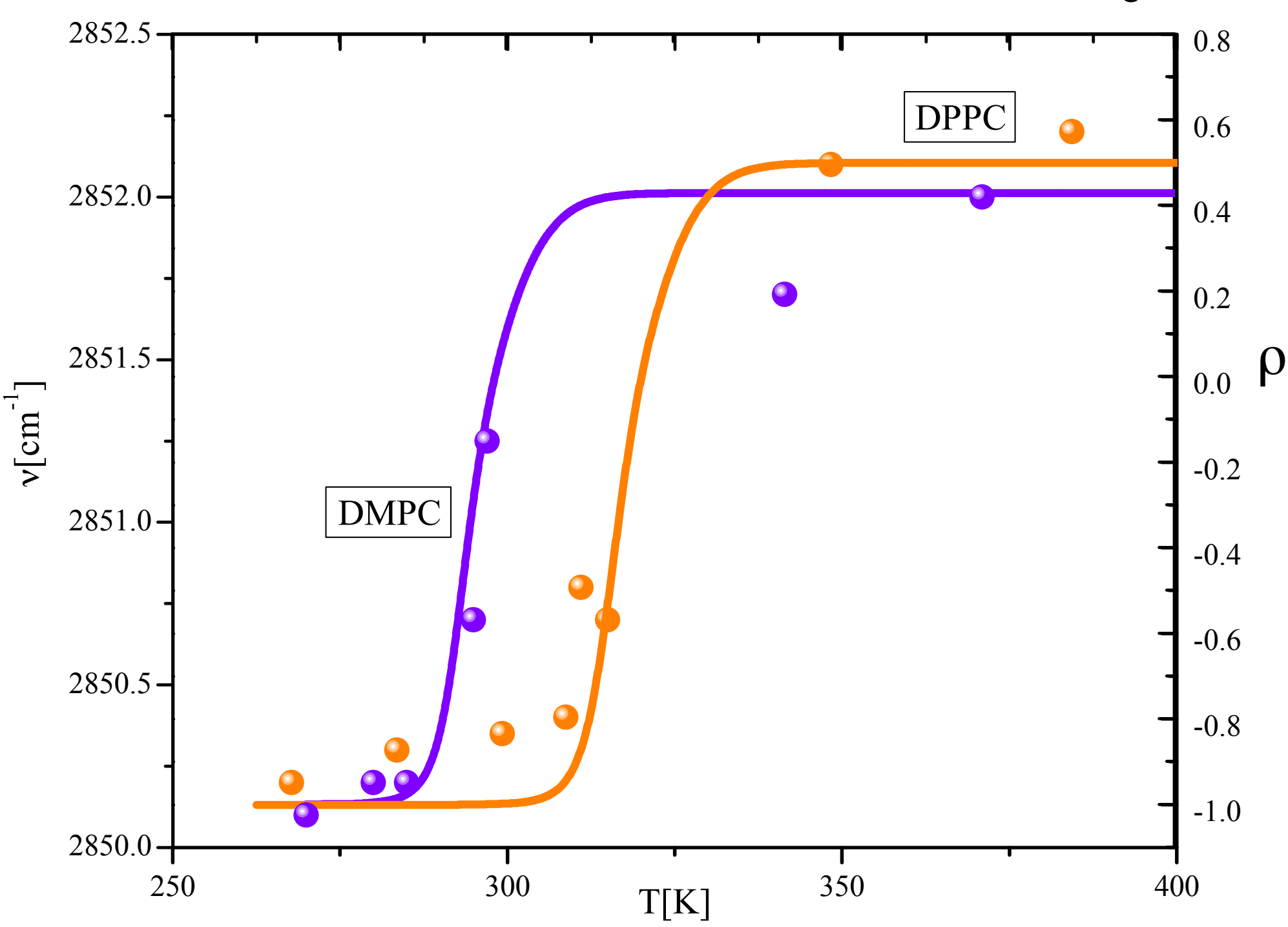


Figure 6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2851.5
2851.0
2850.5
2850.0
ν[cm-1]
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300
350
400
T[K]
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
ρ
DPPC
DMPC