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Melting temperature and heat of fusion of cytosine revealed from fast scanning calorimetry

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ABSTRACT

Thermophysical properties in the melting range of cytosine, one of the five nucleobases of DNA and RNA, are hard to determine because of the low thermal stability of the compound and the high vapor pressure. As for other biomolecules fast heating rates allow melting of cytosine without detectable decomposition. By applying fast scanning calorimetry with the heating rate at 6000 K s^{-1} we succeeded to avoid decomposition and determine the melting temperature of cytosine (extrapolated to zero heating rate), as T_{fus} = (606 ± 4) K, the glass transition temperature of the supercooled liquid state as T_g = (388 ± 3) K, cold-crystallization temperature as T_{cryst} = (448 ± 8) K, and the liquid state molar heat capacity $C^{\circ}_{p,m}(l)$ = (272 ± 2) J mol^{-1} K^{-1} at 423 K. Taking into account the temperature dependent mass loss of the nanogram sized sample (up to 25% during the melting scan) we obtained the molar enthalpy of fusion of cytosine as $\Delta^{l}_{cr}H(T_{fus})$ = (35 ± 4) kJ mol^{-1} in good agreement with the adjusted molar enthalpy of crystallization $\Delta^{cr}_{l}H(T_{fus})$ = (34 ± 2) kJ mol^{-1}.

1. Introduction

Cytosine is a derivative of pyrimidine and one of the four nucleobases which form the main core of Deoxyribonucleic acid (DNA). The four nitro-containing nucleobases cytosine (C), adenine (A), thymine (T) and guanine (G) hold the whole genetic instruction used in growth, development, reproduction and functioning of the organism and even viruses. The particular chemical nature of nucleobases allows for the precise replication of DNA molecules during the cell division process [1–3]. Cytosine, for example, is a relatively small molecule (MW 111.1 g mol^{-1}) however it contains several functional groups (a carbonyl oxygen, primary amine nitrogen, secondary amine ring nitrogen, and a double bonded ring nitrogen), which determine the hydrogen-bonding within its crystal structure [4]. Compounds based on cytosine, such as citicoline [5,6], cytarabine [7,8], and 5-azacytidine [9] have found pharmacological applications. The chemical industry produces a diverse range of pyrimidine derivatives which find application in the pharmaceutical, agrochemical and dye industries [10,11].

Nowadays, computational approaches are widely used for simulation and prediction of the behavior of biological systems starting from small biomolecules [12–15] up to living cell [16,17]. Those thermodynamic data allow for further development and improvement of processes in biopharmaceutical engineering [18–20]. Nevertheless, computational approaches require critical assessments regarding their validity by experimental thermophysical data.

Thermodynamic data on the melting behavior are of high practical importance for the development of pharmaceuticals [18–20]. Modeling approaches were developed for the estimation of melting and solubility properties of organic compounds [21–23]. Such models, however, are only accurate within the range of experimentally studied compounds used for training [24]. Cytosine as a molecule containing several functional groups and as a parent compound for a number of pharmaceutical products therefore represents an important compound indispensable for the model-training datasets.

Cytosine, as other nucleobases, commonly decomposes during melting [25–27]. The thermal properties of cytosine in the liquid state are therefore not available. In fact, thermal studies of cytosine or other biomolecules using slow heating rates are often accompanied by thermal decomposition during melting and possible sublimation/evaporation at high temperatures. These processes are making the

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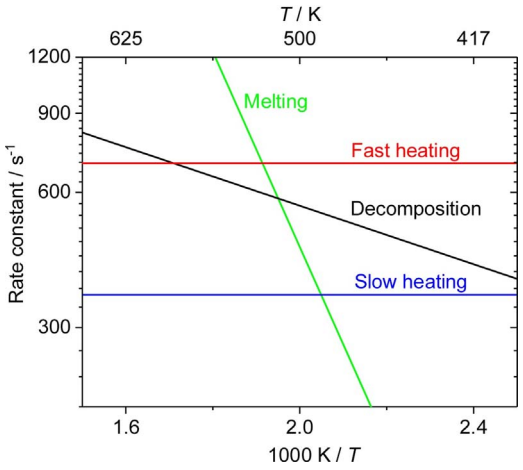

Fig. 1. Schematic activation diagram for melting and decomposition in comparison with slow and fast heating.

estimation of the corresponding thermodynamic parameters, like melting temperature or enthalpy of fusion, highly inaccurate or even impossible. The development of experimental methods, which allow the determination of such properties of biomolecules, is therefore important but an experimental challenge.

To better perceive how temperature affects the structure of DNA, RNA and nucleic acids in general, researchers characterize how their different structural elements affect the thermal stability of these biopolymers [28,29]. It has been found for example, that the thermal behavior of DNA is strongly affected by the sequence length [30,31] and the relative number of cytosine (C) and guanine (G) bases [32–35], and these results denote the important role of the subunits of nucleic acids.

It is therefore of great importance to provide thermodynamic reference data for bio-relevant compounds. Such data help in developing effective computational protocols, however, the real biological processes take place in the liquid (aqueous) state while thermodynamic data for nucleobases are available only in the solid state.

Assuming a certain melting kinetics (superheating) with a constant apparent activation energy allows drawing a melting line in an activation diagram, Fig. 1. For polymers, the apparent activation energy of melting may be up to 5000 kJ mol^{-1} [36]. Typical apparent activation energies for decomposition of polymers are of the order of 200 kJ mol^{-1} [37]. Consequently, both lines are crossing and there is a region in the activation diagram where melting occurs at lower temperature than decomposition. Employing fast enough scanning rates, as accessible in fast scanning calorimetry (FSC), provide a means to avoid thermal decomposition before and during melting. This strategy was already successfully employed for melting of bio-polymers like silk fibroin [38–40] and for low molecular mass compounds like ascorbic acid and prednisolone [41].

Fast scanning calorimetry requires samples of 100 ng or less, depending on the required heating and cooling rates. For such small samples, mass loss due to evaporation or sublimation becomes significant at elevated temperatures. On the one hand this fortunately, opens the possibility to determine enthalpies of vaporization of very low volatile compounds, like ionic liquids [42,43]. On the other hand, the small sample size unfortunately causes serious problems for the determination of the enthalpy of fusion of low molecular mass biomolecules, as discussed below.

In the first part of this paper, on the example of cytosine, we demonstrate the applicability of fast scanning calorimetry for the determination of the melting temperature of a thermally labile and highly volatile bio-relevant molecule. After successfully melting and avoiding degradation by employing a heating rate of 6000 K s^{-1}, immediate cooling at high enough cooling rates yields the amorphous glassy state of cytosine. On successive heating, at 1000 K s^{-1} the glass transition and cold crystallization are seen and the corresponding temperatures are available for the first time. In the second part of the paper, we deal with the determination of sample mass and quantities like specific enthalpy of fusion and specific heat capacity of liquid cytosine. Significant mass loss of the sample during melting aggravates the determination of these mass-specific quantities. Nevertheless, for cytosine we finally succeed in determining the melting temperature of 606 ± 4 K, the glass transition temperature T_g = 388 ± 3 K, the molar enthalpy of fusion of 35 ± 4 kJ mol^{-1} and the liquid state molar heat capacity, $C^{\circ}_{p,m}(l)$ = (272 ± 42) J mol^{-1} K^{-1} at 423 K.

2. Experimental

2.1. Material

The cytosine (Acros Organics, with certified purity > 99%, CAS: 71-30-7) and anthracene (Sigma-Aldrich, with certified purity ≥ 99%, CAS: 120-12-7) were of commercial origin and used without further purification.

2.2. Fast scanning calorimeter

Fast scanning calorimetry employs thin film chip sensors with sub μJ K^{-1} addenda heat capacities [44,45]. These devices allow for controlled cooling and heating at rates up to MK s^{-1} [46,47]. A Mettler Toledo Flash DSC1 [48] was employed for studying the thermal behavior of cytosine during melting. A tiny sample of crystalline cytosine (ca. 10 ng) was placed in the center of the measuring area of an UFS1 sensor [49] (circular area of 500 μm diameter) with the help of a thin copper wire (ϕ = 0.03 mm). In preliminary experiments, the empty sensor was conditioned according to the manufacturer's procedure to establish the correct relation between the measured signal and the temperature of the sample [50]. Temperature and heat flow rate calibrations were finally performed according to the GEFTA recommendation [51] using indium, tin and zinc for the temperature calibration. The FSC scans were performed under an inert atmosphere of nitrogen with a flow rate of about 50 ml/min.

Optical images of the samples under study were taken with an Olympus BX41 microscope with 10 × lens in reflection mode and a DCM 510 camera before and after each thermal scanning, Fig. 2.

Beside temperature and heat flow rate calibration, the measured heat flow rate in chip calorimetry needs additional corrections. The differential chip sensors are commonly very symmetric. Nevertheless, residuals from previously measured samples may introduce some asymmetry, which needs to be corrected by subtracting an empty sensor measurement from the sample measurement.

Contrary to DSC measurements in fast scanning chip calorimetry, the empty sensor measurement does not allow a correction for different heat losses to the surrounding between sample and reference sensor [52,53]. In chip calorimetry, part of the heat exchange between the sensor and the surroundings goes through the sample as schematically shown in Fig. 3 with Φ_3. Consequently, the heat losses depend on the sample properties and cannot be determined independent of the sample.

The only possibility to obtain the required correction is an estimate of the heat losses at a given temperature from the measured heat flow rates themselves. Correction requires heating and cooling scans at the same scanning rate in a temperature region outside any transition region and correction for the empty sensor curves. For a stable sample the absolute values of the heat flow rates are then the superposition of the contributions from the heat capacity of the sample and the differential heat losses [54]. Under the idealized conditions mentioned above, the heat losses depend on the temperature only and they should be the same on heating and cooling. For the measured heat flow rates follows: