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Ice nucleation by water-soluble macromolecules

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Abstract

Cloud glaciation is critically important for the global radiation budget (albedo) and for initiation of precipitation. But the freezing of pure water droplets requires cooling to temperatures as low as 235 K. Freezing at higher temperatures requires the presence of an ice nucleator, which is a foreign body in the water that functions as a template for arranging water molecules in an ice-like manner. It is often assumed that these ice nucleators have to be insoluble particles. We put in perspective that also dissolved single macromolecules can induce ice nucleation: they are several nanometers in size, which is also the size range of the necessary critical cluster. As the critical cluster size is temperature-dependent, we see a correlation between the size of such ice nucleating macromolecules and the ice nucleation temperature. Such ice nucleating macromolecules have been already found in many different biological species and are as manifold in their chemistry. Therefore, we additionally compare them to each other, based on a composition of former, recent and yet unpublished studies. Combining these data with calculations from *Classical Nucleation Theory*, we want to foster a more molecular view of ice nucleation among scientists.

1 Introduction

Although ice is thermodynamically favored over liquid water at temperatures below 273.15 K, the phase transition is kinetically hindered. Consequently, supercooled water stays liquid, until ice nucleation takes place. Homogeneous ice nucleation (see Fig. 1a) is very unlikely, until temperatures as low as 235 K are reached. At higher temperatures, catalytic surfaces which act as an ice-mimicking template are necessary. The process, in which water molecules are stabilized in an ice-like arrangement by an impurity, is called heterogeneous ice nucleation (see Fig. 1b and c). An impurity that possesses this ability is called ice nucleator (IN), or sometimes as ice nucleus. The driving force that causes ice nucleation activity (INA) is the interaction between the partial

charges on the H and O atoms in the water molecules and the properly arranged (partial) charges on the surface of the IN. Therefore, the IN has to carry functional groups at the proper position to be effective (Liou et al., 2000, Zachariassen and Kristiansen, 2000). In most cases it is not the whole surface of an IN that participates in ice nucleation, but only certain sections, which are known as “active sites” (Edwards et al., 1962; Katz, 1962).

The larger the active site of an IN, and the more fitting functional groups it carries, the more effective it stabilizes ice clusters, and so the higher the freezing temperature. Consequently, single molecules of low-molecular compounds cannot nucleate ice. In fact, soluble compounds consisting of very small molecules or ions, like salts, sugars or short-chained alcohols, cause a freezing point depression. However, if single molecules are so large that they allocate enough active surface, they are INs by themselves. Such ice nucleating macromolecules (INMs) are especially common among biological INs. Due to the same reason some low-molecular organic compounds which show no INA in solution can act as IN, if they are crystallized in layers of a certain arrangement (Fukuta, 1966). More considerations about the ice nucleation process are presented in Sects. S1.2, S1.3, and S1.4 in the Supplement.

INA has been discovered among a variety of organisms, including certain bacteria, fungi, algae, plants and animals. Studies to characterize the active sites of some of these organisms have revealed in almost all cases that they are biopolymers. The chemistry of these INMs is as diverse as the range of species they represent: Overall, proteins, higher saccharides and lipids can play a role in INA (see Table 1). In the case of bacteria, it is a certain class of proteins. The known bacterial INMs (BINMs) are fully sequenced and characterized (e.g. Abe et al., 1989), while more questions remain unresolved concerning the other biological INMs. In some cases, biological INMs of one type or species show more than one freezing temperature in an ice nucleation spectrum. This can be explained by the presence of different functional groups, different foldings or aggregation states, which also differ in their INA (e.g. Govindarajan and Lindow, 1988a; Augustin et al., 2013; Dreischmeier et al., 2014; this study). The presence

of INMs seems to have certain advantages, which might be the motivations for certain species to produce them (see Sect. S1.5 in the Supplement).

The bacterial gene is highly conserved and codes for a 120 kDa β-helical membrane protein with many repeated octapeptides (Green and Warren, 1985; Abe et al., 1989; Kajava and Lindow, 1993; Schmid et al., 1997; Graether and Jia, 2001; Garnham et al., 2011). The INA induced by this protein also involves glycosides and lipids that stabilize it in the outer membrane of the bacterial cell and assure its conformation for an optimum functioning (Kozloff et al., 1984; Govindarajan and Lindow, 1988a; Turner et al., 1991; Kawahara, 2002). With the side chains, the total mass of a single BINM is about 150–180 kDa (see Table 1). It is assumed that the initiation point for ice formation is the amino acid sequence TXT in the repeated octapeptide, where *T* designates threonine and *X* any other amino acid. The OH groups of the two threonine moieties match the position of oxygen atoms in the ice lattice. Since a BINM contains several of these sequences at positions and distances that correspond to the ice lattice structure it can stabilize an ice embryo and so decrease the activation barrier for ice nucleation (Graether and Jia, 2001). As sequence modification studies on a structurally related antifreeze protein have shown, the loss of the TXT has a devastating effect on the interaction with water molecules, while other modifications have a much weaker impact (Graether et al., 2000).

The existence of such BINMs has been reported for several species of γ-Proteobacteria, such as a wide range of strains in the *Pseudomonas syringae* species complex (Lindow et al., 1982; Berge et al. 2014); *Ps. fluorescens* and *borealis* (Fall and Schnell, 1985; Obata et al., 1987; Foreman et al., 2013); *Erwinia uredovora* (Obata et al., 1990a); *Pantoea agglomerans*, formerly called *E. herbicola* (Phelps et al., 1986,); *Pant. ananatis* (Coutinho and Venter, 2009); *Xanthomonas campestris* (Kim et al., 1987); a *Pseudoxanthomonas* sp. isolated from clouds (Joly et al., 2013); and more. The efficacy of their INA depends on the strain, as well as on the cultural growth conditions, e.g. the amount of accessible nutrients and the growth temperature (Rogers et al., 1987; Nemecek-Marshall et al., 1993; Fall and Fall, 1998). In most cases, these

BINMs are aggregated and anchored in the outer cell membrane, where the strength of the INA depends on the aggregation state and the chemistry of the membrane (Govindarajan and Lindow, 1988a, b; Kozloff et al., 1991). However, BINMs that have been isolated from the cell membrane show still appreciable INA, although less than in the native state (Schmid et al., 1997). Since these complexes match the ice crystal lattice perfectly, these bacteria are the most active IN known at present.

These anchored aggregates of BINMs on the otherwise ice nucleation inactive cell surface are a demonstrative example of active sites on a larger IN, i.e. the whole bacterial cell which is about 1 µm long. In some cases, bacteria release cell-free INs that are carried on particles that are only a small fraction of the size of the cell. This is the result of the formation of membrane vesicles, spherical pieces of the outer cellular membrane that are excised from the cell, a natural and common phenomenon in bacteria in general (Deatherage and Cookson, 2012). The expression of such vesicles with BINMs has been reported for *Pant. agglomerans* (formerly *E. herbicola*) (Phelps et al., 1986), *E. uredovora* (Kawahara et al., 1993), and *Ps. fluorescens* (Obata et al., 1993). For the production of BINM-carrying vesicles by *Ps. syringae* and *viridiflava* special culture conditions are necessary (Obata et al., 1990b; Pooley and Brown, 1990). For *Ps. putida*, the INA found in culture supernatants was associated with a 164 kDa lipoglycoprotein and had activity both as an IN and as an antifreeze protein. In contrast to the BINMs from the species described above, removal of the approximately 92 kDa of carbohydrates eliminated the INA. The antifreeze properties, however, were not affected (Xu et al., 1998).

INMs were also found in the kingdom of fungi. Similarly to the bacteria, only a limited fraction of investigated strains showed INA, while the majority was inactive (Pouleur et al., 1992; Tsumuki et al., 1995; Iannone et al., 2011; Pummer et al., 2013a; Huffman et al., 2013; Fröhlich-Nowoisky et al., 2014). Species that showed appreciable INA in laboratory studies include *Fusarium* sp. (Pouleur et al., 1992; Hasegawa et al., 1994; Tsumuki and Konno, 1994; Tsumuki et al., 1995; Richard et al., 1996; Humphreys et al., 2001), lichen mycobionts (Kieft, 1988; Kieft and Ahmadjian, 1989; Kieft and

Ruscetti, 1990), rust fungi (Morris et al., 2013; Haga et al., 2013), *Mortierella alpina* (Fröhlich-Nowoisky et al., 2014), *Acremonium implicatum* and *Isaria farinosa* (Huffman et al., 2013). The characterization of the last two INMs is a part of this study. Fungal INMs can be divided into two subgroups, both of which differ from the BINMs. The INMs of rust fungi show properties of polysaccharide compounds (Morris et al., 2013), while the others are evidently proteins. The already characterized INMs from the lichen *Rhizoplaca chrysoleuca* (Kieft and Ruscetti, 1990), from *F. avenaceum* (Pouleur et al., 1992; Hasegawa et al., 1994; Tsumuki and Konno, 1994), and from *M. alpina* (Fröhlich-Nowoisky et al., 2014) barely showed similarities with BINMs, apart from being proteinaceous. For example, they are more tolerant to stresses, have a different amino acid sequence, seem to have less to no lipid and carbohydrate functionalizing, and are extracellular, since they pass through filters with submicrometer pores. Only recently, a 49 kDa protein from *F. acuminatum* was suggested as being the INM (Lagzian et al., 2014). The study also suggests that posttranslational functionalization takes place in the native state and improves the INA, which is a new finding in comparison to former studies (Kieft and Ruscetti, 1990; Tsumuki and Konno, 1994; Fröhlich-Nowoisky et al., 2014).

INs were also found in extracellular fluids of multicellular organisms. The larvae of *Tipula trivittata* (a crane fly) carry an INA-positive 800 kDa lipoprotein in their hemolymph, which shares a high similarity with the BINMs (Duman et al., 1985, 1991; Neven et al., 1989; Warren and Wolber, 1991). The hemolymph of the queens of *Vespula maculata* (a hornet) contains a 74 kDa hydrophilic INA protein (Duman et al., 1984), and the hemolymph of *Dendroides canadensis* (fire-colored beetle) larvae contains a cocktail of an INA protein, an INA lipoprotein and an antifreeze protein (Olsen and Duman, 1997). Most of the known animal INs are proteinaceous, although there are some exceptions, such as the calcium phosphate spherules and fat cells in the larvae of *Eurosta solidaginis* (a gall fly) (Mugnano et al. 1996). INs have also been detected in other animal taxa, e.g. amphibians (Wolanczyk et al., 1990) and mollusks

(Aunaas, 1982; Hayes and Loomis, 1985; Madison et al., 1991; Lundheim, 1997), as well as in spider silk (Murase et al., 2001).

The fluid reservoirs of some succulent plants, namely *Lobelia telekii* and *Opuntia* species, contain polysaccharide INMs (Krog et al., 1979; Goldstein and Nobel, 1991, 1994). Other non-proteinaceous INs have also been found in plants such as the ones reported from the wood of *Prunus* species (drupes) (Gross et al., 1988), or the lignin in a waste water sample (Gao et al., 1999). Only few plant INs, like those of *Secale cereale* (winter rye, Brush et al., 1994), have been clearly identified as proteins. The pollen of some plant species showed appreciable INA in different lab studies, among which that of silver birch (*Betula pendula* or *alba*) was the most active one (Diehl et al., 2001, 2002; von Blohn et al., 2005; Pummer et al., 2012; Augustin et al., 2013). All pollen with INA that were further investigated produce easily extractable INMs, but apart from that showed some differences from each other. As it was confirmed by vibrational spectroscopy, the extracts of pollen contain saccharides, lipids, proteins, and in some cases carotenoids, but no signature of sporopollenin, which is the sturdy hydrophobic polymer building up the outer pollen wall (Pummer et al., 2013b). Birch pollen INMs have a size between 100 and 300 kDa, are tolerant to dry heat (up to 450 K), to high acid and guanidinium concentrations, as well as to several enzymes. Overall, they show typical non-protein and non-lipid behavior (Pummer et al., 2012).

Fungi are abundant and diverse in the atmosphere (Fröhlich-Nowoisky et al., 2009, 2012). Therefore, their potential for atmospheric ice nucleation has to be regarded. In this study, the INMs that were recently found in *A. implicatum* and *I. farinosa* were characterized and compared to other biological INMs, especially the recently characterized INA proteins in *M. alpina* (Fröhlich-Nowoisky et al., 2014). We also expand our knowledge about the chemistry of the birch pollen INMs (Pummer et al., 2012).

2 Methods

2.1 Characterization of new fungal INMs

The fungi *A. implicatum* and *I. farinosa* were cultivated on a plate of potato dextrose agar (VWR™), incubated at ambient temperature for 1–2 weeks, until the first mycelium was formed, and then left to grow at ~ 280 K for 2–3 months (*A. implicatum*) or 6–10 months (*I. farinosa*). The mycelium was scratched off with a scalpel or an inoculating loop and put into a 15 mL Falcon tube. Then 10 mL high-purity water (18.2 MΩ cm) was added, which was tapped from a water purification system (Thermoscientific™ Barnstead GenPure xCAD plus), autoclaved at 394 K for 20 min, and at last filtrated through a sterile 0.1 µm PES filter (Corning™). The suspension was then shaken with a vortex device (VWR™ lab dancer) three times for 30 s and filtrated through a 5 µm PES syringe filter (Acrodisc®), yielding a transparent solution. A small aliquot of the 5 µm filtrate was branched off for INA measurement as described later in this chapter, while the rest was further filtrated through a 0.1 µm PES syringe filter (Acrodisc®). A small aliquot of the 0.1 µm filtrate was saved for later INA tests. Further aliquots were exposed to different procedures, which are listed below, and then tested for their INA. The change of INA provides information about the chemistry of the INMs. In all cases, not only the filtrates but also pure water samples which were treated the same way were tested as a negative reference.

- Filtration through size exclusion filtration tubes (Vivaspin® 500): 300 and 100 kDa cutoff. The passage through a filter indicates that the molecules are smaller than the given cutoff.
- Exposure to heat for 1 h: 308 and 333 K, providing information about the thermal stability.
- Addition of 6.0 M guanidinium chloride (Promega®), which is a chaotropic reagent used for protein denaturation.

- Addition of 0.3 M boric acid (National Diagnostics®), which esterifies with saccharide OH groups and so blocks the site.
- Digestion with enzymes (Applichem®) for at a given incubation temperature: Lipase for 1 h at 308 K for fat digestion, papain for 5 h at 296 K for protein digestion. For the latter, two more temperatures were investigated (5 h at 308 K, 1 h at 333 K), since its optimum temperature is about 338 K, but the investigated INMs turned out to be rather thermolabile. Conveniently, papain still functions at far lower than its optimum temperature, but with lower reaction rates. In our case, the lowest investigated temperature was sufficient.

To determine the IN concentration per gram of mycelium, each sample was diluted with ultrapure water to its proper dilution (which was determined by trial and error) according to Eq. (1). Then, 50 µL aliquots of the dilute were pipetted into 24–32 wells of a 96 well PCR tray (Axon™), which was then sealed with adhesive foil. The plate was then inserted into an isolated PCR-plate thermal block, which was tempered by a cooling bath (Julabo™ Presto A30). For recording a nucleation spectrum, the block was cooled to an initial temperature of 269.15 or 270.15 K. Then the block was further cooled in 0.5 to 2 K steps each 12 min. After each step, the number of frozen droplets was counted. They can be discriminated from liquid droplets, since they reflect the incident light differently, and so appear much darker. We calculated the IN concentration (number of INs per grams of mycelium) via a variant of the Vali formula (see Eq. 1, Vali, 1971):

$$n_m \left[g^{-1}\right] = -\ln(1 - f_{\text{ice}}) \cdot \frac{V_{\text{wash}}}{V_{\text{drop}}} \cdot \frac{F_{\text{dil}}}{m_{\text{myc}}} \quad (1)$$

f_{ice} is the fraction of frozen droplets, V_{wash} the volume of water added for washing (10 mL in this study), V_{drop} the droplet volume in the freezing assay (0.05 mL in this study), F_{dil} the dilution factor of the extract and m_{myc} the mass of the mycelium. For the formula to work, a proper dilution, where $0 < f_{\text{ice}} < 1$ is fulfilled, is necessary. In case of $f_{\text{ice}} = 0$, the dilution is too high, and the formula gives $n_m = 0$ as a result. In case of $f_{\text{ice}} = 1$, the

sample is too concentrated, since n_m becomes infinite. It is mentionable that the Vali formula has a slight inaccuracy, since it assumes that a freezing droplet contains only one IN. However, the distribution of INMs in the droplets follows Poisson statistics, so even at low concentrations some droplets may contain two or more INMs (Augustin et al., 2013).

To quantify the efficacy of the new-found INMs of *A. implicatum* and *I. farinosa* in comparison with others, we used the *Soccer Ball Model* (Niedermeier et al., 2011, 2014), which combines *Classical Nucleation Theory* with the assumption of a contact angle distribution to calculate mean contact angles θ and standard deviations σ from the 0.1 µm filtrate curves. Via a mass-to-size conversion table for proteins by Erickson (2009), we estimated the diameter of our INMs to be about 4 nm, which was used for the *Soccer Ball Model* parameterization. In comparison, we also calculated mean θ and σ of *M. alpina* from comparable filtrates (Fröhlich-Nowoisky et al., 2014), and added literature data for birch pollen INMs (Augustin et al., 2013) and BINM (Niedermeier et al., 2014). Although the concept of contact angles was originally developed for conventional ice nucleating particles, the application on INMs works perfectly. In fact, one can assume that from the mechanism, there is no difference between INA of a free INM and INA on a heterogeneous surface.

INA was also measured with two more systems. For both setups, 0.1 µm filtrates that were prepared as described at the top of this chapter were properly diluted and applied. Resulting values for n_m are compared to the n_m derived from the conventional freezing droplet array.

1. A freezing droplet array called "Bielefeld Ice Nucleation ARraY" (BINARY), which consists of a 6 × 6 array of microliter droplets on a hydrophobic glass slide on top of a Peltier cooling stage. A detailed description of the system, the preparation and the measurements is given in Budke and Koop (2014).

2. A vertical flow tube named "Leipzig Aerosol Cloud Interaction Simulator" (LACIS), which is described in detail in Hartmann et al. (2011). Basically, droplets are generated from the filtrate and dried. The residual particles are then size-selected, humidified to form uniform droplets and inserted into the tube, where they are cooled to the temperature of interest. The procedure was similar to that for the birch pollen washing waters described in Augustin et al. (2013).

2.2 Characterization of birch pollen INMs

To test the hypotheses that birch pollen INMs are polysaccharides and no proteins (Pummer et al., 2012), further procedures were carried out to characterize the birch pollen INMs. Therefore, birch pollen extracts were prepared by suspending and shaking 10 mg mL^{-1} pollen in ultrapure water for several hours, and then vacuum filtering the suspension through a 0.1 µm PES filter (Corning™). The aqueous fraction was then exposed to different treatments, and n_m was determined the same way as for the fungi, with 24 or 32 droplets per sample, at 258 or 256 K. In all cases, reference samples without addition of the reagents were measured and defined as 100 % INA. The results are listed in Table 2.

First, an aliquot was spiked with 0.75 M boric acid, left overnight at room temperature, which is known to esterify with sugars. In case that saccharides play a role, this treatment should alter the INA of the birch pollen INMs. However, since the esterification process does not necessarily affect all functional groups, the INA might be only partially eliminated. On the other hand, the INA assay preparation has a certain statistical uncertainty, which makes minor changes in INA difficult to interpret. Therefore, we also investigated untreated birch pollen extracts as a reference. The same procedure was repeated with heating aliquots with and without boric acid to 343 K for 2 h to accelerate the esterification process.

To check if birch pollen INMs are indeed non-proteinaceous, three 100 µL aliquots were prepared as described: (i) 94 µL water added, (ii) 94 µL medium added, (iii) 94 µL medium and trypsin added, and all of them incubated for 18 h at 310 K. Additionally, 100 µL water was treated like (iii). Trypsin is an enzyme that breaks down proteins, but demands a certain medium. For each sample an INA assay as described in Sect. 2.1

was run. To check, if the enzymatic treatment shifts the mass range of the birch pollen INMs, they were separated with a size exclusion chromatography column. Details about the sample preparation and separation are given in Sect. S2.2 in the Supplement.

2.3 INA of BINM peptides

A sample of the 16-amino acid peptide fragment which is the repetitive element in the *Ps. syringae* BINM was investigated for its INA. The peptide with the primary sequence GSTQTAGEESSLTAGY was obtained from PSL (Heidelberg, Germany) and purified chromatographically using a HiTrap Desalting column (GE Healthcare) with high-purity water (18.2 MΩ cm) from a Milli-Q water purification system (Millipore). The yield of pure peptide was determined using a NanoPhotometer (ε_0 = 1490 M^{-1} cm^{-1}).

We measured peptide solutions with 10, 20, and 30 mg mL^{-1} via the oil immersion cryo-microscopic method, which is described in detail in Pummer et al. (2012). Therefore we prepared emulsions consisting of 45 % wt aqueous peptide solution and 55 % wt oil (paraffin-lanolin). The frozen fractions of droplets with diameters of 20–50 µm were documented with the software Minisee© as a function of temperature.

3 Results/discussion

3.1 Characterization studies

The results of the chemical characterization of the fungal filtrates are composed in Fig. 2. The quantitative passage through the 0.1 µm pore size filters, yielding optically transparent, particle-free filtrates, demonstrates that those INMs are cell-free and stay in solution, when they are extracted with water.

The initial freezing temperature was 269 K for *I. farinosa* and 264 K for *A. implicatum*. The calculated contact angles for *I. farinosa* and *M. alpina* are the highest, while the one of *A. implicatum* lies in the range of the BINM one (see Table 1). The reduction

of INA by papain and by guanidinium chloride indicates that the INMs of both species are proteinaceous. Lipids seem to play a role in *A. implicatum*, but none in *I. farinosa*. Both were resistant against boric acids, making a contribution of carbohydrates to the INA unlikely. Both INMs are more heat sensitive than other fungal INMs, since they were already destroyed at 333 K. *A. implicatum* has a mass of 100 to 300 kDa, since it quantitatively passes through the 300 kDa filter, but not through the 100 kDa filter. About 95 % of *I. farinosa* INM were retained in the 300 kDa filter in comparison to the 0.1 µm filter, and the initial freezing temperature is shifted below 268 K. This suggests that there are larger, more active states of *I. farinosa* INMs and smaller ones active at lower temperatures.

Figure 3 shows the comparison between the data from BINARY, LACIS, and the droplet freezing array (see Sect. 2.1). In general, a good agreement can be seen between the data obtained with the different methods. However, it also becomes clear that onset temperatures, which were often reported in the past, do not properly describe the ice nucleation process. They are dependent e.g. on the detection limit of the different measurement methods used, and particularly for small IN concentrations, impurities or droplets which randomly contain a much more than average amount of ice nucleating material can influence these onset temperatures much. Hence, in the following, T_{50}, i.e. the temperature at which 50 % of all droplets froze, will be used. For that value, however, also a note of caution should be given, as droplets with larger concentrations of similar IN will have higher freezing temperatures, due to an increased probability of freezing.

The results of the birch pollen measurements, which are given in Table 2, suggest that both the medium and the boric acid led to a reduction in INA. However, the addition of trypsin had no additional effect at all, which speaks against a proteinaceous nature of those INMs. It is most likely that it is the formic acid that decreases the INA in the medium, since it esterifies with hydroxyls similar to the boric acid. This is consistent with the resistance against other proteases and guanidinium chloride (Pummer et al., 2012), and the lack of the spectroscopic signature typical for proteins in the most active

eluates. Overall, we confirm that the birch pollen INMs are no proteins, but most likely polysaccharides.

After applying the SEC column, the INMs were spread across the whole eluate. This might be explained by the glue-like behavior of the birch pollen extract (Pummer et al., 2013b), which causes adhesion to the packing material and therefore undermines the separation principle of the column. Nevertheless, there was an unambiguous maximum in the 335 to 860 kDa fraction before and after digestion. This is the more intriguing, since we recorded the absorbance of the eluate at 280 nm via a UV detector, which is a quite reliable way to detect most proteins. However, the detector showed no signal when the INA maximum was eluted. This alone would make it very unlikely that the birch pollen INMs are proteinaceous. The discrepancy with the mass range stated by Pummer et al. (2012) could be explained by the slightly higher investigation temperatures, which was a necessity of the setup, which corresponds to a larger critical cluster or INM size. We suggest that the birch pollen INMs might be capable of forming aggregates that are larger, active at higher temperatures, but also less frequent. Consequently, they are overseen in INA assay devices with lower material loads per droplet, such as the oil immersion cryo-microscopy.

3.2 Critical cluster size

In the following, we will compare INMs, for which molecular mass and ice nucleation temperature were determined experimentally, with the critical water molecule cluster size, which depends on the temperature. For the latter, we use the parameterization by Zobrist et al. (2007), which is based on *Classical Nucleation Theory*. All available data are put together in Table 3 and Fig. 4. Apart from the fungal and birch pollen INMs investigated in our groups, we added BINM data by Govindarajan and Lindow (1988a), who already indicated the good agreement between aggregate size and critical cluster size. INA data of polyvinyl alcohol (PVA) were incorporated, since it also showed a slight INA in experiments (Ogawa et al., 2009). Its peculiarities are first that the formula is quite simple for a macromolecule, which is a sequence of CH_2CHOH-units, and

second that the chain is rather randomly coiled. Therefore, the near-range molecular order is quite well defined, while the far-range order is merely statistical.

The data of birch pollen and fungal INMs are in appreciable agreement with the theoretical parameterization. From that we deduce that singular biological INMs which carry a suitable hydration shell are the perfect ice templates, but with the advantage that they do not randomly dissociate like ice embryos in homogeneous ice nucleation. This explains their high INA.

In the case of PVA, we see that an increase in size does not lead to an appropriate increase in the freezing temperature. This can be easily explained by the different degrees of structure of biological macromolecules and technical homopolymers. Both PVA and BINMs consist of a sequence of monomers covalently linked to each other, like the wagons of a train. As the backbone shows some flexibility, longer chains will not be bolt upright sticks, but fold into more compact three-dimensional structures. Without any further forces, polymers coil randomly, like a string of wool that tends to ravel. Therefore, confined geometries do not exceed the size of a few monomers, where it is the limited flexibility of the monomer-to-monomer bond that causes confinement. Hence, an increase in the total INM mass will not increase its INA. In contrast, intact proteins have a strongly determined folding, which is held together by intramolecular forces (e.g. hydrogen and disulfide bonds), and sometimes even forced on them by folding-supporting proteins. Therefore, a native protein's structure is stabilized in a certain geometry, as is the molecular surface. The unfolding of a biological macromolecule – a process called denaturation – changes also many of its properties. This is also valid for the INA of INMs, and explains their deactivation by heat far below the temperatures where the covalent molecular bonds are broken. It is also responsible for the destruction of most INMs by the chaotropic guanidinium chloride. Summed up, randomly coiled INMs like PVA allocate only small, one-dimensional templates for ice nucleation (see Fig. 1b) and are therefore rather inefficient. Consequently, the ice nucleation temperatures are maximum a few Kelvin above the homogeneous freezing temperature (see Fig. 4). On the other hand, molecules in confined geometries, like the BINM, allocate

stable two-dimensional surfaces as ice nucleating templates (see Fig. 1c), which are larger and therefore nucleate at higher temperatures (see Fig. 4). Also long-chained alcohols show appreciable INA, if they are crystallized in well-defined monolayers, depending on the chain length, the position of the OH group, and substitutions on the side chains (Popovitz-Biro et al., 1994). Of course, the surface of these 2-D-templates has to be properly functionalized in order to arrange the water molecules, or else they show no INA at all.

3.3 INA of BINM peptides

The examination shows that the 16-amino acid BINM peptide shows INA, when a certain concentration in solution is surpassed. In view of Fig. 4, this molecule should barely show INA, since its molecular mass is only 1.6 kDa and the number of fitting functional groups is limited to one TXT motif. However, these peptides tend to self-assemble into aggregates (Garnham et al. 2011), which consequently follow equilibrium of formation and decay. These aggregates may have different sizes and forms (e.g. parallel versus antiparallel β sheets), and consequently different INAs.

If the fractions of frozen droplets are plotted against the temperature, it can be seen that while the 10 mg mL^{-1} sample showed only homogeneous ice nucleation, the 30 mg mL^{-1} sample showed an initial freezing temperature of about 250 K, from which a broad flat slope ranged down to the homogeneous ice nucleation range. The variance of T_{50}, which ranges from 240 to 245 K in different experiments, is rather high, since the aggregate formation seems to be very sensitive to the handling of the sample. This is in contrast to the typical biological INMs, which show a very steep slope at a given temperature and then reach a saturation plateau (see e.g. Figs. 2 and 3). Further investigations are in progress to measure the aggregates and get a better understanding of the process.

4 Discussion and conclusions

4.1 Basic physics of INA

In atmospheric science, INs are traditionally regarded as insoluble particles on the surface of which ice nucleation takes place. According to Raoult's law, soluble substances are expected to decrease the freezing point with increasing molar concentration. Furthermore, as already stated, the template has to be of a certain size to make ice embryos that are large enough to grow. Consequently, particles that dissociate into low-molecular compounds in solution (e.g. NaCl, mono- and disaccharides) cannot act as IN. However, data by Pummer et al. (2012) showed that the ice nucleation active components of pollen have a mass between 100 and 300 kDa. This means, the INs have the size of single macromolecules. If these molecules are fully dissolved in water, one can regard them as being in solution and not in suspension. Many proteins are soluble in water (e.g. Osborne, 1910; Macedo, 2005; see Sect. S1.1 in the Supplement), but single molecules are far larger than e.g. salt ions or lower sugars. Therefore, a deviation from the simplistic approach of Raoult's law is expectable. In this case, a soluble compound can also be an IN, if the molecular surface is large enough to stabilize ice embryos. The freezing point depression is expected to be rather weak for a dissolved > 100 kDa molecule, because even a high mass concentration correlates with only a low molar concentration. The resulting small reduction of the solution water activity is likely to affect the heterogeneous ice nucleation temperature only slightly (see Sect. S1.4 in the Supplement, Koop and Zobrist, 2009; Attard et al., 2012). Accordingly, certain macromolecules can act as IN in spite of being water-soluble, because the water-structuring effect over-compensates the colligative freezing point depression. Most molecules carry a well-defined hydration shell. In case of INMs, the geometry of water molecules in the hydration shell is supposedly similar to the geometry in an ice embryo, what triggers the freezing process (see Fig. 1). We therefore emphasize that a more molecular view on IN allows better understanding. We see the link between this

molecular view and the macroscopic view that is necessary for atmospheric models in the contact angles.

As shown in Fig. 4, molecular size and INA exhibit a positive correlation. Deviations from the model line can be explained by different properties of different types of INMs. If molecules are larger than expected, like the birch pollen INMs, the active site might not be the whole molecule, but just a small part of it. The INMs of *I. farinosa* and *M. alpina* seem to be too small. This can be either explained by spontaneous aggregation of several molecules after the filtration step, or by a large hydration shell around these INMs that has to be added to the total IN mass. Also, when data were derived from measurements in which droplets were examined which contain higher numbers of INM per droplet, the freezing temperature is shifted to higher temperatures, as can e.g. be seen when comparing data of birch pollen from Pummer et al. (2012) and Augustin et al. (2013). Very speculatively, one could try to go the other way and use experimentally determined freezing temperatures of IN, e.g. mineral dust and soot, to roughly estimate the size of their active sites. In combination with chemical and structural analyzing of the IN, one could try to identify which elements of these IN can be considered to be responsible for the INA. Considerations about the INA and active sites of mineral dust are given in Sect. S1.6 in the Supplement.

4.2 Atmospheric impacts

Apart from its cryobiological and evolutionary aspect, heterogeneous ice nucleation is of high importance for atmospheric research, since it causes cloud glaciation, and therefore impacts the global radiation budget (albedo) and initiates precipitation.

It is a common argument against the atmospheric INA potential of bioaerosols that whole cells that are at least some micrometers in size are far too large to reach altitudes higher than a few kilometers. The detection of cultivable microorganisms even in the mesosphere (Imshenetsky et al., 1978) shows that there have to be mechanisms that elevate intact cells to the higher atmosphere. As an example, the atmospheric turbulences caused by volcanic activity support a high- and far-range distribution of all kinds

of aerosols (van Eaton et al., 2013). Furthermore, certain pollen (e.g. pine) and fungal spores (e.g. urediospores) are very buoyant, as they possess wing-like projections and other aerodynamic surface properties. Urediospores have been collected from the air at over 3 km above the ground level along with other microorganisms (Stakman and Christensen, 1946). Cultivable microorganisms have also been collected from the stratosphere (Griffin, 2004). At last, microorganisms are frequently found in precipitation samples (e.g. Amato et al., 2007), what indicates their presence at cloud formation altitudes. Even more intriguingly, some of these organisms are even able to proliferate in supercooled cloud droplets (e.g. Sattler et al., 2001).

Furthermore, biological cells are not rigid spheres, but rather a composition of many different membranes, organelles and fluids, which further consist of many different molecules, ranging from water to small organic molecules and to biopolymers. Therefore, the release of molecular matter, as well as cell fragmentation, is common. Several studies detected molecular tracers from pollen grains and fungi in atmospheric fine particulate matter even in the absence of whole cells (e.g. Solomon et al., 1983; Yttri et al., 2007). In most cases, biological INMs are easily released from the producing cell (see Table 1). Since a single primary biological particle can carry up to hundreds and thousands of INMs, and since the INMs are also much lighter, we expect their atmospheric concentration to be significantly higher as well. A possible mechanism of INM release is cell rupture caused by a rapid change in moisture. Scanning electron microscopy studies on wet pollen back up this idea by visualizing the release of organelles and organic matter (Grote et al., 2001, 2003; Pummer et al., 2013b). This explains why rainfall, which is expected to wash out aerosols, can indeed increase the concentration of allergens (Schäppi et al., 1999) or INs (Huffman et al., 2013) in the air.

Quantifying the atmospheric impact of fungi is even more difficult, as presumably 1 to 5 million fungal species exist (Hawksworth, 2001). Due to mutation and adaptation, every species consists of numerous strains, which differ in their INA (Tsumuki et al., 1995). Even if all studies are combined, it is only a minor fraction of all fungal species that have been tested for their INA. Furthermore, the expression of INMs is triggered

by yet unknown conditions, which could be the availability of nutrients, the local climate or competition with other microorganisms. As a consequence, INA-positive strains can lose their activity when they are cultivated under laboratory conditions (Tsumuki et al., 1995; Pummer et al., 2013a). Therefore, more atmospheric IN counting and sampling will be necessary to understand the contribution of biological INA better.

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Table 1. The chemical properties of some INMs. "T stability" shows the temperature about which the IN are denatured. An interrogation mark indicates some uncertainty. See Introduction for the sources of these data. $\theta\,[^\circ] \pm \sigma\,[^\circ]$ are the calculated contact angle distribution according to the *Soccer Ball Model*.

Type	Organism	cell-free?	protein?	glycoside?	lipid?	T stability	size (1 unit)	$\theta\,[^\circ] \pm \sigma\,[^\circ]$
BINMs:	*Ps. syringae*	no	yes	yes	yes	< 313 K	150–180 kDa	34.1 ± 2.3
	E. herbicola	yes	yes	yes	yes	< 313 K	150–180 kDa	
Fungal INMs:	*Rhiz. chrysoleuca*	yes	yes	no	no	> 333 K	< 0.22 µm	
	F. avenaceum	yes	yes	no	no	> 333 K	< 0.22 µm	
	A. implicatum	yes	yes	no?	yes	308–333 K	100–300 kDa	33.2 ± 2.3
	I. farinosa	yes	yes	no?	no	308–333 K	~ 300 kDa	24.6 ± 0.6
	M. alpina	yes	yes	no?	no	333–371 K	100–300 kDa	26.4 ± 1.1
	rust spores	??	??	yes	??	~ 373 K	??	
Animal IN:	*Tipula*	yes	yes	yes?	yes	??	800 kDa	
	Dendroides	yes	yes	no?	both	??	> 70 kDa	
	Vespula	yes	yes	no	??	< 373 K	74 kDa	
	*Eurosta**	yes	no	no	no	??	> 100 µm	
Plant IN:	*Secale* leaves	??	yes	yes	yes	< 363 K	??	
	Prunus wood	no	no	??	??	313–323 K	??	
	Betula pollen	yes	no	yes	no	445–460 K	100–300 kDa	58.2 ± 4.6
	Lobelia fluid	yes	no	yes?	no	> 373 K	??	
	Opuntia fluid	yes	no	yes	no	??	< 70 µm	
	Algae	??	??	??	??	??	??	

* Only the calcium phosphate spherules are regarded here, not the fat cells.

Table 2. An overview over the investigation on birch pollen extracts. The percentage is the relative number of INs in comparison to the untreated aliquot at a given temperature T [K]. Lines labeled with "(ref)" refer to reference measurements under the same conditions with pure water instead of extract.

Treatment	% INA	T [K]
none	100	both
none (ref)	< 9	both
boric acid	15	256
boric acid (ref)	0	256
343 K	29	256
343 K + boric acid	3	256
medium	34	258
medium + trypsin	30	258
medium + trypsin (ref)	13	258

Table 3. Overview over masses (m) and activation temperatures (T_{nuc}) of certain IN.

Type	Source	m [kDa]	T_{nuc} [K]
BINM (∼ 560 units)	Burke and Lindow (1990)	∼ 83 700	272
BINM (∼ 130 units)	Govindarajan and Lindow (1988a)	∼ 19 000	271
BINM (∼ 60 units)	Govindarajan and Lindow (1988a)	∼ 8700	270
BINM (∼ 20 units)	Govindarajan and Lindow (1988a)	∼ 2500	268
ice embryo	Zachariassen and Kristiansen (2000)	810	268
Isa-INM (> 1 units)	this study	> 300	268
Isa-INM (1 unit?)	this study	100–300	267
Mor-INM	Fröhlich-Nowoisky et al. (2014)	100–300	266
BINM (3 units)	Gurian-Sherman and Lindow (1995)	∼ 360	263
BINM (1 unit)	Govindarajan and Lindow (1988a)	∼ 150	261
INAFP	Xu et al. (1998)	164	261
Acr-INM	this study	100–300	259
birch INM	this study	335–860	257
birch INM	Pummer et al. (2012)	100–300	255
birch INM*	Augustin et al. (2013)	100–300	250
PVA	Ogawa et al. (2009)	1.7–98	239
ice embryo	Zachariassen and Kristiansen (2000)	1.26	233

* T_{nuc} here are T_{50} of both the LACIS measurement with 800 nm particles and the oil immersion cryo-microscopy measurement with 5 µg mL^{-1} pollen.

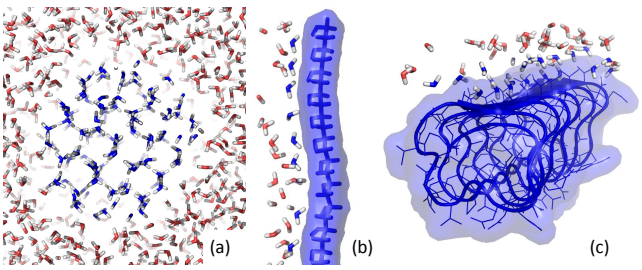

Figure 1. Visualization of water molecule ordering based on molecular model calculations (see Sect. S2.1 in the Supplement): homogeneous ice nucleation **(a)**; heterogeneous ice nucleation by ordering of water molecules on a PVA strain, which is a 1-D-template **(b)**, and an antifreeze protein related to the BINMs, which is a 2-D-template **(c)**. Each image contains water molecules that are ordered (blue) and some randomly distributed water molecules (red).

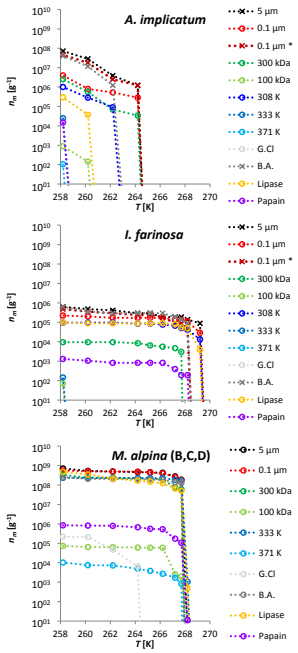

Figure 2. Concentrations of *A. implicatum*, *I. farinosa*, and *M. alpina* (B,C,D) INMs after several treatments. "G.Cl" stands for guanidinium chloride treatment, "B.A." for boric acid treatment. A reduction in n_m suggests that this method partly or fully destroyed the INMs. The data point symbols o and x shall discriminate between different harvests. For *M. alpina*, the data are the mean curves of all investigated strains of the phylogenetic subgroups B, C, and D (Fröhlich et al., 2014). Subgroup A was ruled out due to its resistance against papain. The absence of a curve in a diagram means that no droplets were frozen at all.

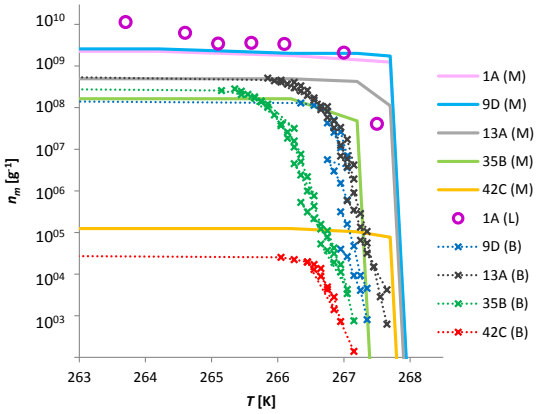

Figure 3. Comparison of ice nucleation curves of 0.1 µm filtrates from a few *M. alpina* strains measured with the droplet freezing assay at MPIC (M), LACIS (L), and BINARY (B).

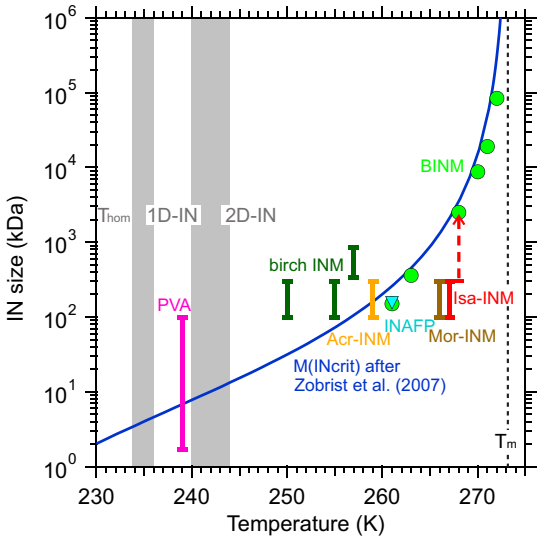

Figure 4. The dependence of the median freezing temperature on the size for different types of IN (colored dots). The blue curve is the calculated critical ice cluster size derived from *Classical Nucleation Theory* (Zobrist et al., 2007). The sources of the presented IN data are listed in Table 3. The graph further shows the region where we assume the domains where 1-D- and 2-D-templates act as IN. The acronyms *Acr*, *Isa*, and *Mor* stand for the respective fungal species.