Elucidating structure-function relationships of amphiphilic copolymer excipients to enhance the stability of biopharmaceuticals

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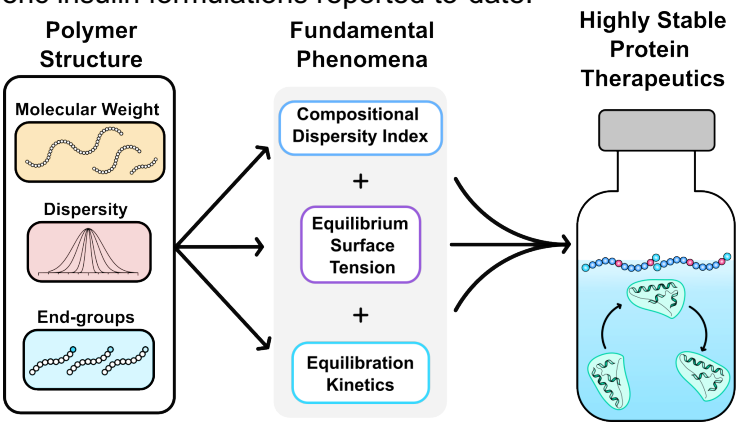
Abstract

Protein therapeutics, or biopharmaceuticals, are the fastest growing class of drugs in the healthcare industry with products currently licensed for a wide range of diseases including diabetes, cancer, and viral infections. Unfortunately, the highly specific tertiary structure that endows biopharmaceuticals with their remarkable efficacy and safety, also significantly limits shelf-stability and ultimately clinical translation. Recently, we validated that the high-throughput screening of chemically distinct polyacrylamide derivatives could yield libraries of surfactant excipients (biologically inert additives), which dramatically improve the stability of biopharmaceuticals including insulin and monoclonal antibodies by over 50-fold compared to standard commercial surfactants. Here, we explore and isolate the impact of fundamental polymer properties on the stability of clinically relevant ultra-fast acting (monomeric) insulin. A library of compositionally identical polymers was synthesized, and optimization of molecular weight, dispersity, and end-group functionality doubled the stability of monomeric insulin formulations compared to our previous reports, which was ultimately 5-fold more stable than commercial fast acting insulin drug products. Most importantly, with the diverse structure-function relationships present in this new library, we were able to quantify, validate, and predict the fundamental phenomena responsible for the performance of these copolymer excipients. Overall, we present a rigorous investigation into the structure-function relationships of amphiphilic polyacrylamides, validating a framework for the rational design of next-generation excipients and engineering the most stable monomeric insulin formulations reported to-date.



Introduction

Biopharmaceuticals are the fastest growing class of drugs in the healthcare industry, with the number of products on the market increasing from 13 in 1989 to over 435 in 2022.[1,2] While the pace of product approvals remained steady until 2015, the rate has tripled from 2017 to 2022, due to the success of antibody and peptide-based therapies increasing the market value from $188 billion to over $343 billion.[2] The success of these types of biologics is due in large part to complex and delicate tertiary structures that endow these drugs with highly potent and specific biological activity. Unfortunately, these same complex structures make biologics unstable at ambient conditions, necessitating cold chain storage and formulation optimization for clinical translation. “Non-active” additive technologies, called excipients, have been crucial for the translation of lifesaving drugs from the bench to the bedside.[3] As proteins are inherently amphiphilic, many are highly prone to aggregation induced by hydrophobic interfaces, such as air or silanized glass, impairing stability and efficacy.[4,5] Surfactants such as polysorbates and poloxamers are FDA approved excipients (present in >50% of biologics) with amphiphilic properties that form protective layers at these hydrophobic interfaces, improving shelf-life without impacting efficacy.[6] However, newly discovered degradation pathways, safety concerns, and allergic responses have highlighted the critical need to find alternative next generation excipients without cleavable esters[7–9] or polyethylene glycol (PEG) [10,11] functionalities.

Recently, we validated that the high-throughput combinatorial synthesis of amphiphilic copolymers could yield diverse libraries of surfactant excipients, enabling identification and optimization to dramatically improve the stability of biopharmaceuticals, including insulin and antibodies.[4,5,12,13] The use of controlled radical polymerization techniques allowed for the facile combination of 11 chemically distinct monomers into 90 unique copolymers with a constant degree of polymerization. By screening this library with insulin stability assays, a specific polymer composition was isolated that acted as a potent biopharmaceutical excipient, improving formulation stability over 50-fold compared to commercial surfactant excipients. Crucially, this

polyacrylamide derivative addressed the key issues facing current commercially available excipients. The copolymer surfactant is composed of highly stable amide bonds, does not contain PEG, and exhibits potent surfactant action while not forming micelles.[4,8,9] This work was one of the first examples of screening polymer composition to discover new functionality, contributing to the now growing field of high-throughput copolymer discovery.[14–21] These ongoing efforts have seen tremendous success by leveraging the vast chemical space that can be realized by simply combining chemically distinct monomers at different compositional ratios; however, other polymer properties such as molecular weight, dispersity, and end-group functionality have been neglected in these screening investigations. There is a growing appreciation for the importance of these seemingly minor polymer characteristics, and indeed end-group functionality has recently been highlighted as a driver of polymer-protein and polymer-interface interactions. [22–28] Moreover, new studies which simulate individual polymer chains highlight that even within systems of compositionally identical polymers, there can be significant differences between structure and function.[21] With regards to polyacrylamide excipients, there exists a unique opportunity to further characterize the impact of molecular weight, dispersity, and end-group functionality on protein stability and to elucidate mechanisms behind these emergent structure-function relationships.

Based on our previous work, we found that a copolymer comprising of acryloylmorpholine (Morph; Mo) and N-isopropylacrylamide (Nipam; Ni) at weight percents of 77% and 23% ($MoNi_{23\%}$) were highly effective at stabilizing multiple protein drugs including insulin and antibodies in aqueous formulations under stressed aging conditions. Considering recent efforts to understand polymer structure-function relationships, we hypothesized that elucidating the connections between polymer molecular weight, dispersity, and end-group functionality on protein stability, would enable rational design of superior next-generation excipients (Figure 1). To evaluate the effect of these properties on excipient performance, we leveraged the versatility of reversible addition fragmentation chain transfer (RAFT) polymerization to synthesize a library of compositionally identical polymers and quantified their impact on the stability of monomeric

insulin, a notoriously unstable biopharmaceutical. Each of these properties plays an important role in the performance of the polyacrylamide excipient and through optimization we were able to more than double the stability of previously reported monomeric insulin formulations under stressed aging conditions. Furthermore, these distinct copolymers enabled rigorous mechanistic investigations, where we discovered that a combination of three properties: equilibrium surface tension, equilibration kinetics, and a new polymer property we call the compositional dispersity index (CDI), can accurately explain and predict the increased performance of these polyacrylamide excipients. We believe this work generates important design criteria for the development of next-generation excipients by providing quantitative and mechanistic insights into polymer structure-function relationships that dramatically improve the stability of biopharmaceuticals.

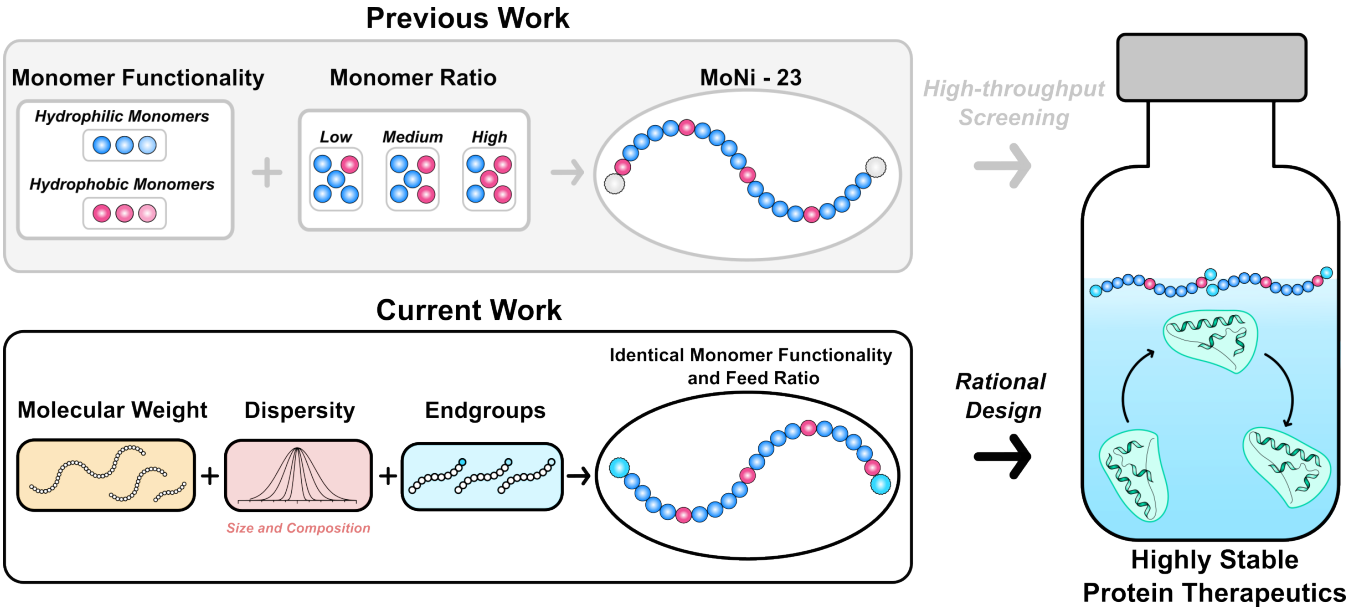


Figure 1. Summary of previous and current polymer property optimization approaches to improve the stability of monomeric insulin formulations.

Results and Discussion

Synthesis of MoNi$_{23\%}$ analogs. We previously established that a MoNi$_{23\%}$ copolymer with a molecular weight of approximately 7 kDa effectively partitions to the air-water interface, precludes protein adsorption at these interfaces, and thereby stabilizes the proteins against

aggregation.[4,5,12,13] To understand the impact of polymer molecular weight, we prepared several copolymers with Mo and Ni incorporation at the previously optimized $MoNi_{23\%}$ composition but doped in increasing amounts of chain transfer agent (CTA) (Figure 2A). For these polymerizations we used 2-cyano-2-propyl dodecyl trithiocarbonate (CPDT) as the CTA, which results in low dispersity polymers (Đ < 1.2) even at long reaction times (> 6 h) or low molecular weights (Table 1). Due to the high fidelity of RAFT polymerization, we were able to synthesize six $MoNi_{23\%}$ polymer derivatives with identical monomer compositions (Figure 2B), but molecular weights spanning an order of magnitude (2 kDa to 20 kDa) according to size exclusion chromatography (SEC) (Figure 2C). We limited the molecular weights we evaluated to 20 kDa because neutral polymers with molecular weights greater than 25 kDa are too large to be excreted renally by kidney filtration.[29] While there is significant overlap of the monomer functionalities and the backbone signals in ^{1}H NMR, we were able to confirm that all reactions proceeded to high conversion and did not have quantifiable differences in chemical composition (Table 1). Lastly, prior to any stability studies, the CTA end-group was removed via an end-group removal reaction with excess azobisisobutyronitrile (AIBN) and lauroyl peroxide (LPO).[4,5,12,13]

Table 1. Summary of $MoNi_{23\%}$ characteristics as a function of molecular weight. Counts of Mo and Ni determined from conversion. Conversion* and M_n* determined with ^{1}H NMR. M_n and M_w determined via SEC with polymethylmethacrylate (PMMA) standards.

Label	Mo	Ni	Conversion	M_n	M_n	M_w	Dispersity
2	11	4	98%	2,400	2,000	2,100	1.08
4	23	8	97%	4,500	3,600	4,000	1.11
8	46	17	97%	8,700	7,600	8,400	1.11
12	69	25	98%	12,900	11,600	13,300	1.14
16	92	34	96%	17,100	13,700	16,200	1.19
20	115	42	97%	21,300	17,500	21,200	1.20
8-HD	33	12	98%	6,000	6,100	9,800	1.62

Impact of Molecular Weight. To study the effect of these $MoNi_{23\%}$ analogs on protein stability, we made slight modifications to previously published assays to increase throughput and

accentuate the differences between polymers.[4] In brief, we used the zinc chelating agent, ethylenediaminetetracetic acid (EDTA), at a concentration of 1.2 mM to convert hexameric commercial insulin into insulin monomers and added polymeric excipients at 0.2 mg/mL (0.02 wt %) before heating to 37 ˚C and shaking continuously. Insulin aggregation was measured by monitoring non-specific absorbance at 540 nm with the time to 10% of maximum absorbance being used as the onset of aggregation (T10 aggregation time).[30] All polymers stabilized monomeric insulin formulations for longer than the commercially available hexameric insulin formulation, trade name Humalog (Figure 2D). Surprisingly, we found that intermediate molecular weights (4 kDa and 8 kDa) stabilized the biopharmaceutical longer than both the low (2 kDa) and high molecular weight (12 kDa, 16 kDa, and 20 kDa) polymers (Figure 2D). While this result explains the success of the original $MoNi_{23\%}$ (7 kDa), it was unexpected because literature precedent implies that surface properties of polymeric surfactants vary linearly with molecular weight, either improving at low molecular weights due to increased packing efficiency or at higher molecular weights due to avidity effects.[27,31] This unique phenomenon of an optimal insulin stability at an intermediate molecular weight hinted at the possibility that our current understanding of surfactant induced stabilization of proteins is incomplete, and that several material properties may be important for this application.

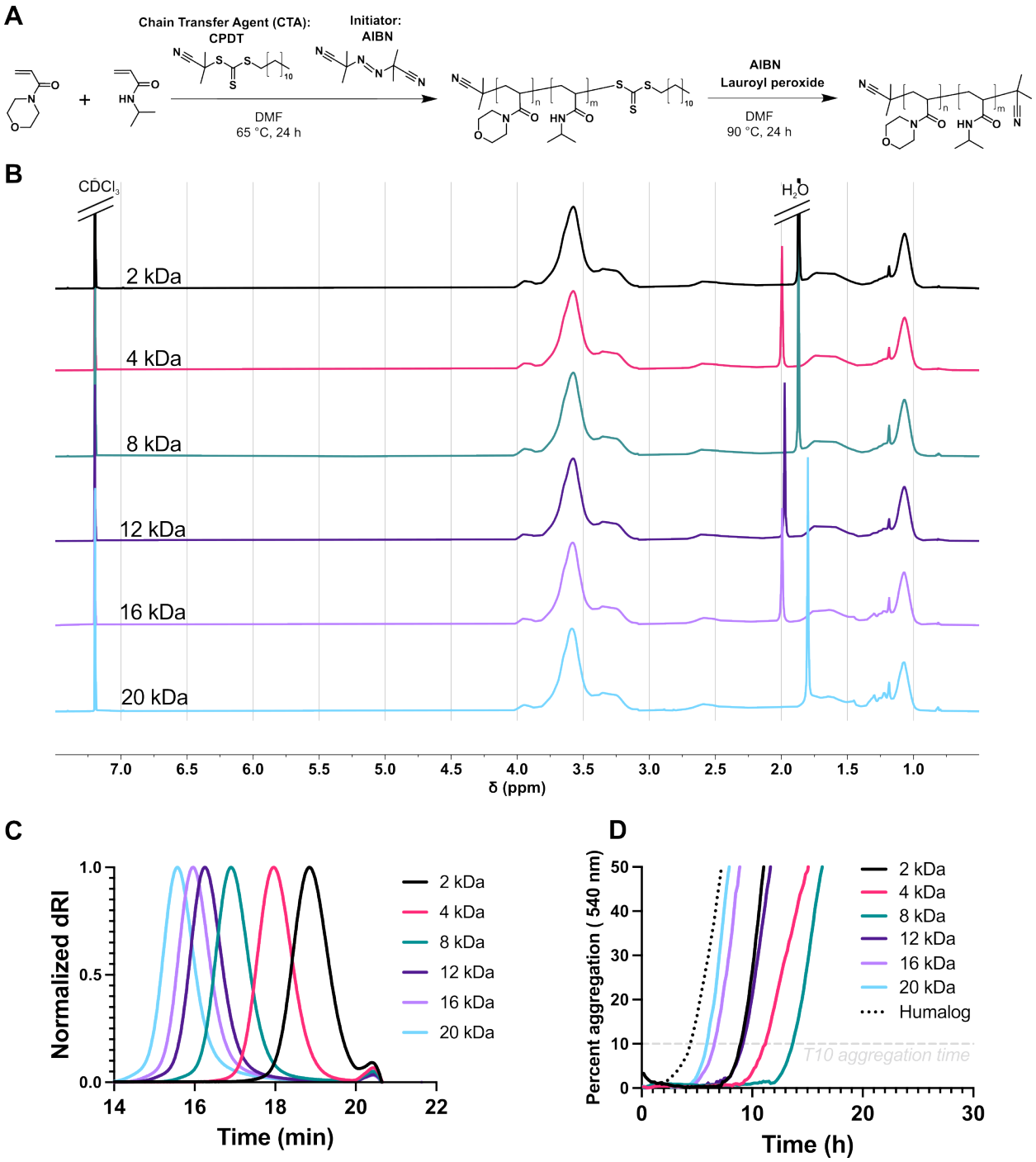


Figure 2. Synthesis, characterization, and evaluation of insulin stability with poly(acrylyoylmorpholine-*co*-N-isopropylacrylamide ($MoNi_{23\%}$) excipients at various molecular weights. A) Synthesis scheme for $MoNi_{23\%}$ including RAFT polymerization and end-group removal. B) ^1H NMR characterization of purified $MoNi_{23\%}$ polymers of various molecular weights in $CDCl_3$. C) Size exclusion chromatography (SEC) determination of $MoNi_{23\%}$ molecular weight and dispersity relative to PMMA standards in DMF with LiBr. D) Non-specific absorbance assay to assess stability under stressed aging conditions (37 ˚C and shaking) of monomeric insulin with EDTA (1.2 mM) + polymer excipients (0.02 wt %) and commercial insulin hexamer (Humalog). Data presented are a mean of 4 technical replicates.

Impact of Molecular Weight Dispersity. As a secondary method to validate the observed effect of molecular weight on insulin stability, we varied polymer dispersity at a fixed molecular weight. If the most "active" components of the polyacrylamide-based surfactant are the polymer chains with the moderate molecular weights (in the range of 4-8 kDa), we hypothesized that at higher dispersities, where these weight fractions will be lower total percentages of the polymer, the insulin stability will decrease. To synthesize high dispersity (HD) polyacrylamides, Anastasaki and coworkers have demonstrated that 2-Cyanopropan-2-yl *N*-methyl-*N*(pyridin-4-yl)carbamodithioate (CMCD) could be used to achieve a target molecular weight with high chain-end retention.[32,33] We replicated these protocols to synthesize compositionally identical 4 kDa and 8 kDa $MoNi_{23\%}$ analogs with dispersities of 1.33 and 1.62 respectively (Figure 3A and S1A). The end-groups of the HD polymers were removed via several radical reactions to ensure complete removal of the CTA (Table 2 and Table S1, Figure S2-5) and mixed with the low dispersity counterparts at various ratios to create new polymers with similar number average molecular weights but varied dispersities (Figure 3B and Table 2). We observed consistent, quantifiable, and incremental decreases in the T10 aggregation time of monomeric insulin as a function of $MoNi_{23\%}$ dispersity (Figure 3C), which was consistent for both 8 kDa and 4 kDa polymers (Figure S1B). These results highlight that while polymers may appear identical at the bulk level (composition and molecular weight) differences at the level of the individual chains in the population (dispersity) can result in distinct functional behaviors. This report is the first to systematically quantify the effect of polymer dispersity in the context of biomaterial optimization and demonstrate its importance as a tunable property.

Table 2. Evaluation of dispersity of $MoNi_{23\%}$ 8 kDa and 8-HD mixtures with SEC and PMMA standards. End-group removal was determined by ratio of the area under the curve (AUC) before and after the reaction at 310 nm.

Label	M_n	M_w	Dispersity	End group removal
8	7,575	8,433	1.11	98.3%
1.20	7,639	9,127	1.20	---
1.26	7,441	9,345	1.26	---
1.29	7,357	9,499	1.29	---
1.33	7,226	9,607	1.33	---
1.40	7,071	9,919	1.40	---
8-HD	6,072	9,815	1.62	98.47%

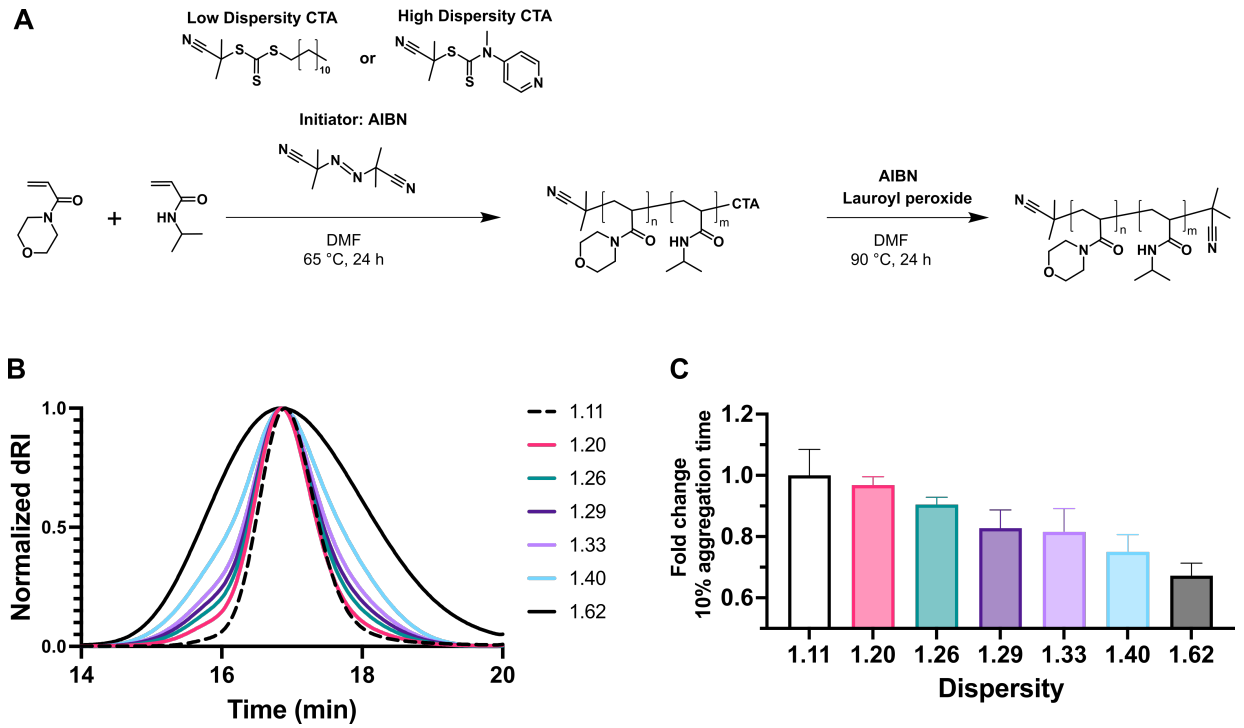


Figure 3. Synthesis, characterization, and evaluation of insulin stability with $MoNi_{23\%}$ excipients at various dispersities. A) Synthesis scheme for 8 kDa $MoNi_{23\%}$ polymers with identical compositions but different dispersities. B) SEC traces for mixtures of high and low dispersity $MoNi_{23\%}$ polymers yielding a gradient of dispersities. C) Comparative T10 insulin aggregation times relative to low dispersity (Đ=1.11) 8 kDa $MoNi_{23\%}$ as a function of dispersity. Data presented is a mean of 4 technical replicates.

Impact of End Groups. To investigate the effect of excipient chain ends on insulin stability, we chose to first study the best performing low molecular weight polymer ($MoNi_{23\%}$ 4 kDa) since we believed the greater proportion of end-groups would magnify the differences between various chemical functionalities. For all $MoNi_{23\%}$ analogs synthesized to this point, the end group removal reaction was performed with AIBN, in accordance with previously established protocols.[4,5,12,13] We continued to leverage the high efficiency of excess radical end-group removal reactions to synthesize new MoNi analogs with 2,2'-Azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70), 2,2'-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044), and 4,4′-Azobis(4-cyanopentanoic acid) (ACVA) and compare their activity to 4 KDa $MoNi_{23\%}$ with AIBN. These initiators cover a range of chemical functionalities at physiologic pH, with V-70 being more hydrophobic than AIBN, ACVA adopting a negative charge, and VA-044 adopting a positive charge (Figure 4A). While all end-group removal reactions achieved greater than 95% efficiency as quantified via absorbance at 310 nm (Table 3 and Figure S6), the chemical identity of the thermal initiator had a significant impact on the performance of the polymeric excipient.

Table 3. Summary of end-group removal efficiency of $MoNi_{23\%}$ 4 kDa with various initiators as determined by AUC at 310 nm during SEC.

Label	End group removal
4-AIBN	99.2%
4-ACVA	99.1%
4-V70	98.9%
4-VA	98.0%
8-AIBN	98.3%
8-V70	95.9 %

Specifically, the neutral initiator V-70 dramatically increased the T10 aggregation time of the 4 kDa $MoNi_{23\%}$ by more than 1.5-fold (Figure 4B and S7). To investigate possible mechanisms and understand the impact of end-group identity as a function of molecular weight, all six polymers

from previous experiments were functionalized with V-70. Contrary to our hypothesis, we observed that the switch from AIBN to V-70 increased the T10 aggregation time of the high molecular weight polymers by 2-fold, proportionally more than their low molecular weight counterparts, which only saw increases of ~1.5-fold (Figure 4C, 4D, and S10). To underscore the impact of simply modulating the end-capping reagent, we show that by SEC and NMR the 8 kDa $MoNi_{23\%}$ with AIBN and V-70 end-groups are essentially identical (Figure S8 and S9) yet the 8 kDa $MoNi_{23\%}$ with V-70 stabilizes insulin for over 24 h compared to the 8 kDa $MoNi_{23\%}$ with AIBN, which aggregates in 12 h (Figure 4E). These results demonstrate that regardless of molecular weight, the chain-end identity of polymeric surfactants plays a vital role in their performance as stabilizing excipients.

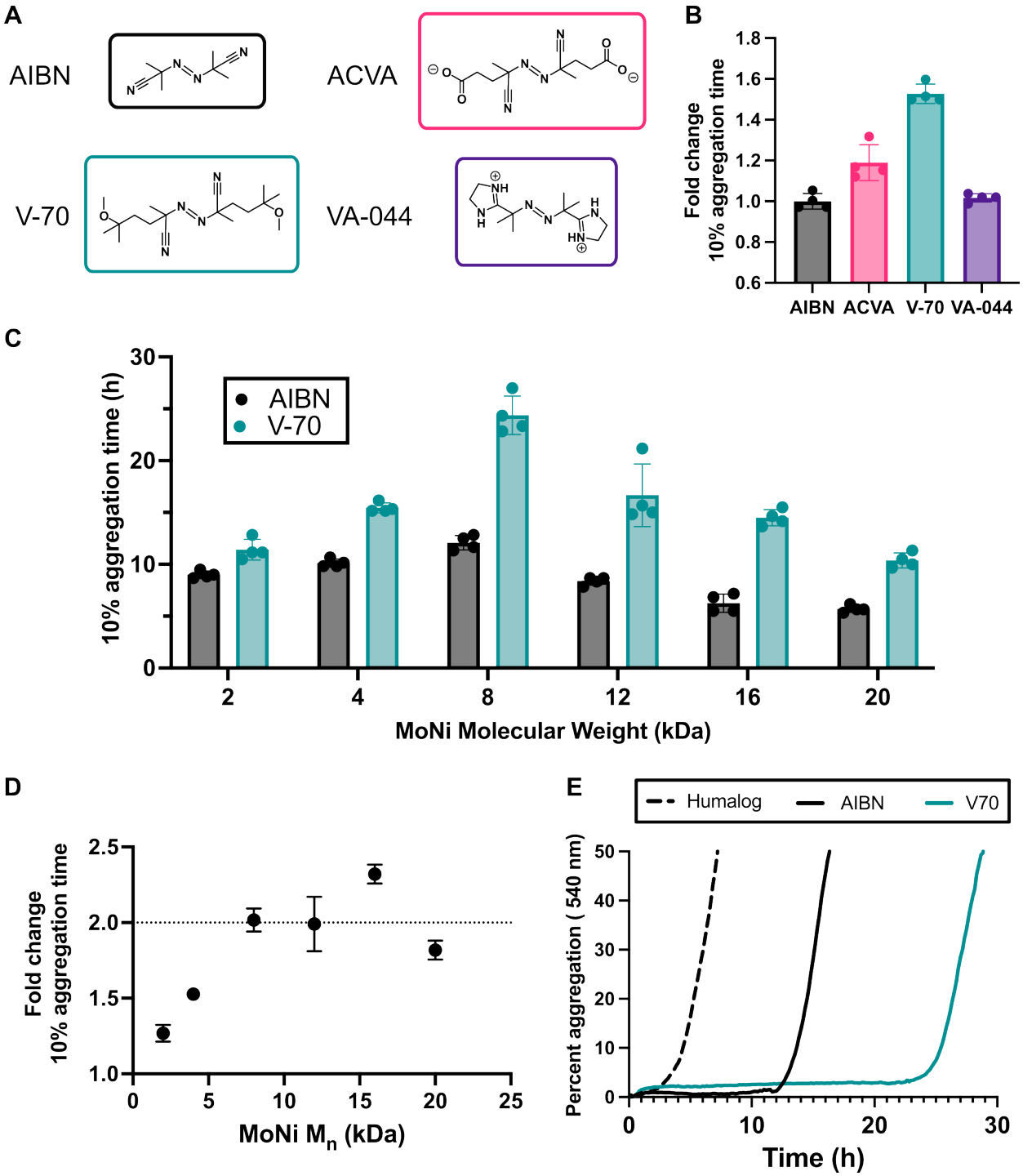


Figure 4. Comparison of various thermal initiators that were used for end-group removal and their impact on insulin stability. A) Structures of thermal initiators and their ionization states in insulin formulations. B) Comparative T10 insulin aggregation times relative to 4 kDa $MoNi_{23\%}$ with AIBN end-groups. C) T10 insulin aggregation times compared at various molecular weights with either AIBN or V-70 end-groups. D) Ratio of T10 insulin aggregation times of V-70 to AIBN end-groups as a function of $MoNi_{23\%}$ molecular weight. E) Representative non-specific absorbance assay of monomeric insulin stabilized with 8 kDa-AIBN and 8 kDa-V70 vs. commercial Humalog. Data presented are a mean of 4 technical replicates.

Mechanistic Investigations of Surface Activity. We previously discovered that for both low and high concentration biopharmaceuticals, $MoNi_{23\%}$ significantly reduced the surface tension of the formulation. These results suggested that the mechanism behind the increased protein stability was a preferential accumulation of polymer at the air-water interface preventing the proteins from denaturing upon interacting with the hydrophobic air.[4,12,13] We suspected that the observed differences in insulin stability as a function of molecular weight, dispersity and end-groups would correspond with lower surface tension values. We chose to evaluate the surface tensions of our best performing polymers (low dispersity with V-70 end-groups) at various molecular weights with the Wilhelmy Plate method (Figure 5A). We observed a monotonic trend in surface tension as a function of the polymer molecular weight with the 2 kDa $MoNi_{23\%}$ equilibrating at the lowest surface tension of 44.88 mN/m and the 20 kDa $MoNi_{23\%}$ equilibrating at the highest surface tension of 52.29 mN/m (Figure 5B). Combined with our previous insulin stability experiments (Figure 4C) demonstrating that the 2 kDa and 20 kDa $MoNi_{23\%}$ polymers have the lowest T10 aggregation time, it becomes clear that surface tension alone is insufficient to explain the performance of polymeric excipients as stabilizers of biopharmaceuticals. Moreover, despite the more than 2-fold difference in T10 aggregation time between 8 kDa $MoNi_{23\%}$ with V-70 and AIBN end-groups (Figure 4E), the equilibrium surface tension of these polymers is almost identical at 49.88 mN/m and 50.92 mN/m (Figure 5C and Table 4). When we plotted the T10 aggregation time vs. surface tension (Figure 5D), no correlation emerged, corroborating our observations that surface tension alone cannot account for increases in protein stability.

While equilibrium surface tension did not yield clear correlations with T10 aggregation times, we noticed differences in the equilibration kinetics during these time-resolved experiments, particularly at short time points (< 5 min, ~300s). We fitted our surface tension curves with a one phase exponential decay curve and extracted rate constants (Table 4 and Figure S11) to quantify how quickly these surfactants partition to the air-water interface. These rate constants roughly

decreased as a function of molecular weight (Figure 5E), with larger polymers achieving equilibrium surface tensions at slower rates than smaller polymers. Similar to the equilibrium surface tension measurements, there was not a simple correlation with protein stability and these rate constants since the smallest 2 kDa $MoNi_{23\%}$ which does not enhance protein stability significantly, had the fastest rate. Yet, when we compared the rate constants for 8 kDa $MoNi_{23\%}$ polymers with either AIBN or V-70 end-groups, the V-70 polymers equilibrated much faster than their AIBN counterparts (Figure 5F and S12). This observation implies that above a certain molecular weight threshold (above 4 kDa for $MoNi_{23\%}$), the faster the surfactant partitions to the air-water interface the greater the enhancement of insulin stability. Importantly, this observation explains why higher molecular weight polymers with slower kinetics benefit more from end group optimization (Figure 4D), as low molecular weight polymers can already achieve equilibrium rapidly due to their small size there is nothing to be gained from the end group substitution.

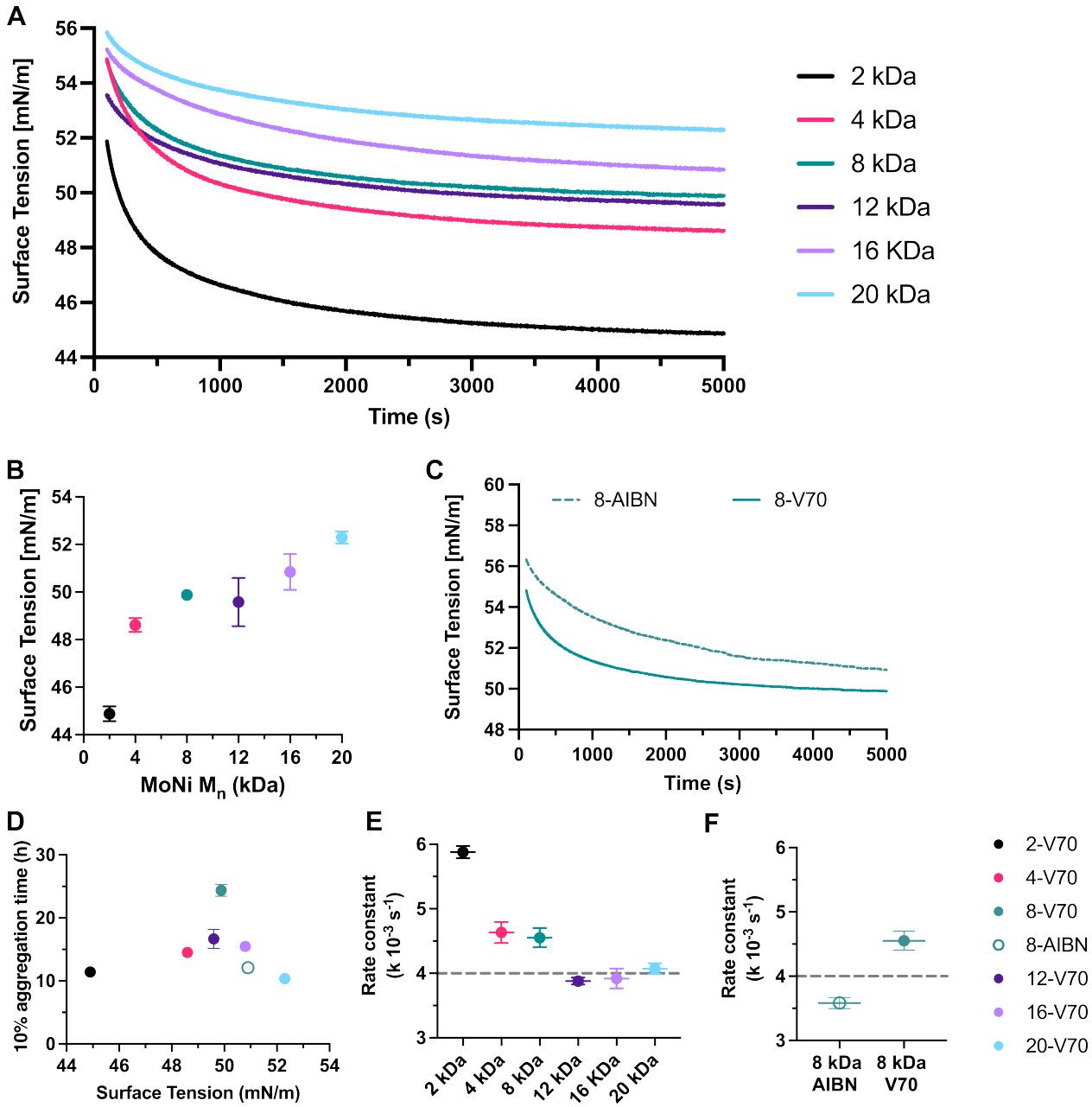


Figure 5. Evaluation of surface tension with Wilhelmy plate method as a function of $MoNi_{23\%}$ molecular weight and end-group identity. A) Mean surface tension values of 0.02 wt % $MoNi_{23\%}$ in Humalog buffer (without insulin) over 5000 seconds (s) (n = 2). B) Equilibrium surface tension of solutions with $MoNi_{23\%}$ excipients (0.02 wt %) at 5000 s as a function of polymer molecular weight. C) Surface tension over 5000 s of 8 kDa $MoNi_{23\%}$ with either AIBN end-group or V-70 end-group. D) T10 aggregation times for insulin stabilized with different $MoNi_{23\%}$ molecular weights as a function of equilibrium surface tension. E) Rate constant for a one phase exponential decay fit of the surface tension equilibration measurements in the first 600 s as a function of $MoNi_{23\%}$ molecular weight. F) Rate constant for a one phase exponential decay fit of the surface tension equilibration measurements in the first 600 s for $MoNi_{23\%}$ with either AIBN end-group or V-70 end-group. Mean and standard error of the mean were obtained from duplicate experiments.

Mechanistic Investigation of Compositional Dispersity. We were struck by the existence of a clear low molecular weight threshold for these polyacrylamide-based excipients, below which, despite excellent surface-active properties, the excipients lost the ability to effectively stabilize insulin. While our polymerization reactions contained identical monomer stoichiometry, the stochastic nature of copolymerizations can lead to a range of possible polymer compositions for each individual polymer chain. Monte Carlo simulations have emerged as a powerful tool to understand the composition of individual chains within a polymerization.[21,34,35] In particular, we noticed that at low molecular weights individual polymer chains deviate substantially from the stoichiometrically ideal copolymer composition.[36] To explore this characteristic among the MoNi variants in our study, monomer ratios, reactivity ratios, and dispersities were used to simulate each polymerization at a given molecular weight with a previously published Monte Carlo simulation package, Compositional Drift (Figures S13-18). We plotted the total degree of polymerization (DP) of each monomer within a single polymer chain to create 2D visualizations of the overall compositional dispersity (Figure 6A). An ideal polymer chain at any given molecular weight would have a ratio of approximately 3 Morph monomers to 1 Nipam monomer, creating a line from the origin with a slope of about 1 over 3 (0.299 to be exact). Therefore, the compositional dispersity of the simulated polymerization can be assessed by how well the distribution of individual polymer chains fits this ideal copolymer line, quantified by R^2. This approach enabled us to quantify the compositional dispersity of each polymer and we found that dispersity decreased at higher molecular weights (higher R^2 values) indicating that more polymers in the simulation achieved a composition close to the ideal.[36] In fact, we observed a similar molecular weight cut-off as in our insulin stability study, due to the dispersity becoming significantly higher below 4kDa. We chose to describe these dispersity values on a scale analogous to molecular weight as compositional dispersity index (CDI, $|1/R^2|$), yielding values of 7.86, 5.54, 2.1, 1.56, 1.25, 1.20 for 2 kDa, 4 kDa, 8 kDa, 12 kDa, 16 kDa and 20 kDa, respectively (Figure 6B). This large spread of individual polymer compositions explains the poor ability of low molecular weight

polyacrylamide-based excipients to stabilize insulin, as our previous work demonstrated that a composition of exactly 77% Morph and 23% Nipam was key to excipient performance.[4] Moreover, at high compositional dispersity, extreme outliers in composition might seed insulin aggregation by inducing amyloid fibril formation in a mechanism similar to short amphiphilic peptides.[37–39] In addition to explaining the inability of short $MoNi_{23\%}$ polymers to effectively prevent protein aggregation, compositional dispersity also explains the slight differences in the performance of 4 kDa and 8 kDa MoNi polymers. Despite almost identical surface properties (Table 4), 8 kDa $MoNi_{23\%}$ stabilizes insulin for 1.5 times longer than 4 kDa $MoNi_{23\%}$. Only when we compare the dispersities, 2.1 vs. 5.54, does it become clear why the 8 kDa $MoNi_{23\%}$ polymer so significantly outperforms its 4 kDa counterpart. In summary, we provide a new metric, CDI, quantifying heterogeneity in the distribution of individual polymer compositions to elucidate subtle differences between polymerizations with identical feed ratios. This metric provides a key insight into the performance of polymeric excipients and when combined with measurements of surface properties can explain and predict the polymer's ability to stabilize protein formulations.

Table 4. Means and standard errors for duplicate measurements of 0.02 wt % solutions of $MoNi_{23\%}$ polymers in Humalog Buffer as measured by the Wilhelmy plate method. Rate constants from one phase exponential decay of surface tension measurements in the first 5 min of equilibration. Compositional Dispersity Index (CDI) for each polymer determined from Compositional Drift simulations. Summary of T10 aggregation times for each polymer evaluated by insulin stability assay.

Label	Equilibrium Surface Tension (mN/m)	Surface Kinetics Rate constant (k 10^{-3} s^{-1})	Compositional Dispersity Index	10% Aggregation Time (h)
2-V70	44.9 ± 0.3	5.878 ± 0.047	7.86	11.42 ± 0.50
4-V70	48.6 ± 0.2	4.633 ± 0.082	5.54	15.46 ± 0.24
8-V70	49.88 ± 0.02	4.551 ± 0.074	2.10	24.37 ± 0.93
12-V70	49.6 ± 0.7	3.880 ± 0.027	1.56	16.67 ± 1.51
16-V70	50.8 ± 0.5	3.919 ± 0.077	1.25	14.5 ± 0.39
20-V70	52.3 ± 0.2	4.070 ± 0.042	1.20	10.37 ± 0.36
8-AIBN	50.9 ± 0.6	3.583 ± 0.044	2.10	12.08 ± 0.35
Humalog Buffer	71.960 ± 0.002	N/A	N/A	N/A

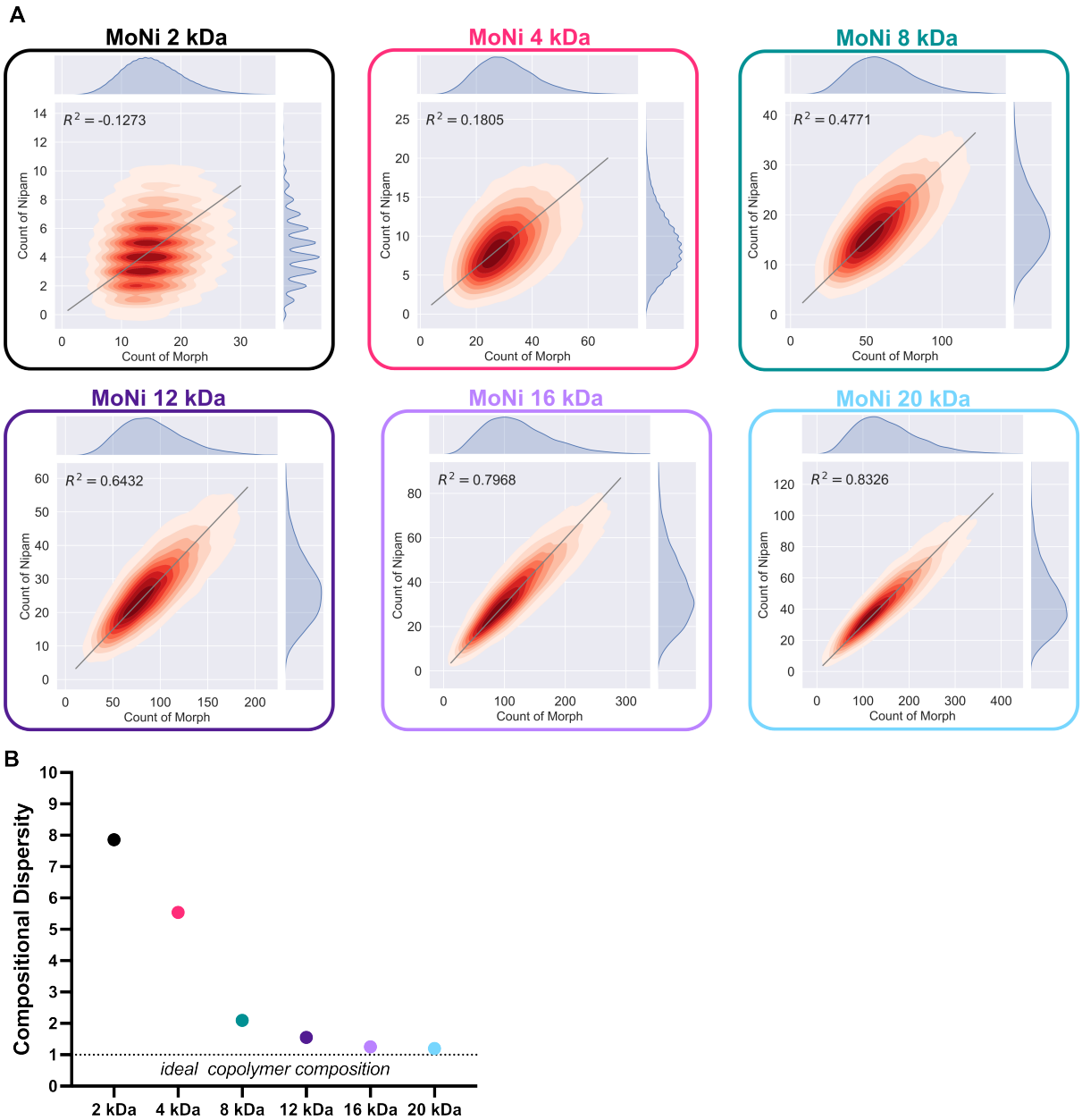


Figure 6. Compositional dispersity plots generated from Compositional Drift simulation. A) 2D-plots of incorporation of Morph and NIPAM simulated for individual polymer chains to visualize monomer compositions of $MoNi_{23\%}$ polymers with various molecular weights. To quantify deviation from the ideal monomer composition based on feed ratio (Morph/NIPAM of 77/23), R^2 was calculated for the distributions against a line with a slope of 23/77. B) Compositional Dispersity Index (defined as $|1/R^2|$) as a function of $MoNi_{23\%}$ molecular weight.

Fundamental Design Principles for Effective Polyacrylamide Excipients. Through our mechanistic investigations, we determined that surface tension, equilibration kinetics, and compositional dispersity index (CDI) can explain the performance of copolymer excipients. With

these factors in mind, we propose a framework for designing copolymer excipients that maximizes protein stability (Figure 7A). First, copolymers must be of sufficient molecular weight to have a homogenous monomer composition at the individual polymer level (low CDI). Second, copolymers must be small enough to pack tightly at the air-water interface (as determined by equilibrium surface tension). Third, given copolymers with similar compositional dispersities and equilibrium surface tensions, the highest rate constant for surface equilibration will provide the most protection against protein aggregation. Using the $MoNi_{23\%}$ library as a case study, we can begin to understand trends in insulin stability as a function of each of these mechanisms (Figure 7B). When we plot CDI vs. a surface property (such as rate constant), clusters with high and low T10 aggregation times emerge. However, with only two properties it is still difficult to account for all the variation observed between polymers. Applying the above framework, we can isolate a range of molecular weight values over which each mechanism plays the dominant role in predicting insulin stability (Figure 7C). At low molecular weights (at and below 8 kDa) CDI explains 99% of the variation in the T10 aggregation time (Figure 7D). At high molecular weights (at or above 12 kDa), equilibrium surface tension explains 99% of the variation in insulin stability (Figure 7E). Finally, at intermediate molecular weights (between 8 kDa and 16 kDa) the rate constant for equilibration explains 96% of the variation in T10 aggregation times, including differences between end-groups (AIBN vs. V70). The ability of this rate constant to predict insulin stability in this group of extremely similar polymers (8 kDa-V70, 12 kDa-V70, 16 kDa-V70, and 8 kDa-AIBN), which all had compositional dispersities below 3 and equilibrium surface tensions values between 49.9 and 50.9 mN/m, is strong evidence that we have developed a rigorous understanding of the fundamental principles underlying polymeric excipient performance. Taken together, we have clearly defined three regimes where CDI, equilibrium surface tension, and equilibration kinetics can explain and predict the performance of copolymer excipients.

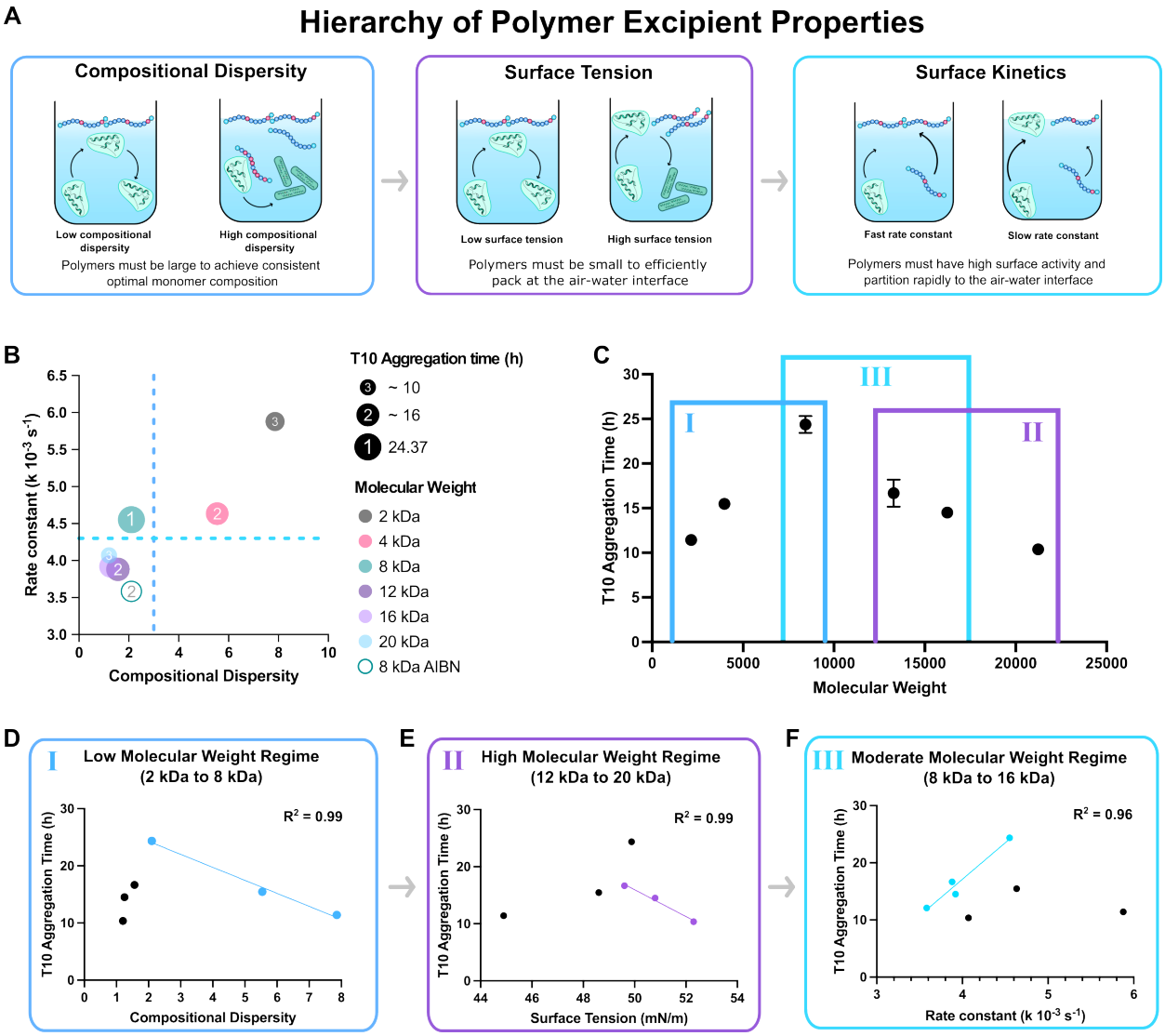


Figure 7. A) Schematic overview summarizing polymer properties and their impact on excipient performance. Polymers must first have low compositional dispersity, then low surface tensions, and finally fast equilibration kinetics to prevent aggregation of monomeric insulin. B) Comparison of rate constants, compositional dispersity, and T10 aggregation time for various $MoNi_{23\%}$ polymers. C) Selection of 3 molecular weight regimes and the key polymer property that explains and predicts excipient performance (T10 aggregation time). D) Linear correlation between Compositional Dispersity Index and T10 aggregation time at low molecular weights. E) Linear correlation between Surface Tension and T10 aggregation time at high molecular weights. F) Linear correlation between rate constant and T10 aggregation time for polymers with low compositional dispersity and low surface tensions (moderate molecular weights). Fits performed with Prism 10.

Conclusion

In this study we demonstrate that polymer properties traditionally important to bulk material behaviors, including molecular weight, molecular weight and compositional dispersity, and end-group functionality, are also key to the performance of excipients in solution. Modular polymeric surfactants present an opportunity to isolate new variables for excipient optimization, especially compared to current commercial excipients such as polysorbates and poloxamers. Since our previous work demonstrated the effectiveness of screening chemical compositions to improve insulin stability, we designed this study to analogously quantify the impact of molecular weight, various types of dispersity, and end-group functionality on excipient performance. Despite identical monomer compositions across the polyacrylamides tested, we observed large variations in the polymeric excipient's ability to stabilize monomeric insulin. Specifically, we found that (i) molecular weight optimization more than doubled the stability of monomeric insulin formulations compared to commercially available "fast-acting" insulins like Humalog (from 5 h to 12 h), (ii) increasing dispersity reduced the T10 aggregation time by almost 40%, and (iii) end-group optimization approximately doubled the T10 aggregation time (from 12 h to 24 h). Additionally, the broad range of structure-function relationships demonstrated by these compositionally identical polymers enabled rigorous investigation of the mechanisms responsible for excipient performance. With quantification of surface activity and the development of a new polymer descriptor, compositional dispersity index (CDI), we established three molecular weight regimes where the stability of monomeric insulin could be linearly explained by a single dominant polymer property. For the first time for this class of excipients, we define a framework that enables accurate prediction of excipient performance from fundamental polymer properties. In summary, we leveraged the distinct structure-function relationships in this library of compositionally identical polymers to dramatically increase the stability of therapeutically relevant monomeric insulin and define the key mechanisms responsible for the performance of amphiphilic copolymer excipients.

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