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Engineering Insulin Cold Chain Resilience to Improve Global Access

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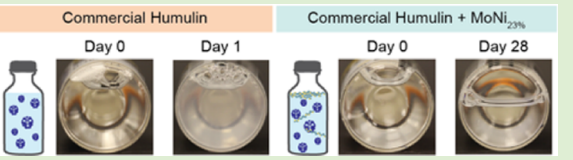
ABSTRACT: There are 150 million people with diabetes worldwide who require insulin replacement therapy, and the prevalence of diabetes is rising the fastest in middle- and low-income countries. The current formulations require costly refrigerated transport and storage to prevent loss of insulin integrity. This study shows the development of simple "drop-in" amphiphilic copolymer excipients to maintain formulation integrity, bioactivity, pharmacokinetics, and pharmacodynamics for over 6 months when subjected to severe stressed aging conditions that cause current commercial formulation to fail in under 2 weeks. Further, when these copolymers are added to Humulin R (Eli Lilly) in original commercial packaging, they prevent insulin aggregation for up to 4 days at 50 °C compared to less than 1 day for Humulin R alone. These copolymers demonstrate promise as simple formulation additives to increase the cold chain resilience of commercial insulin formulations, thereby expanding global access to these critical drugs for treatment of diabetes.



INTRODUCTION

There are over 150 million people with diabetes requiring insulin replacement therapy worldwide.[1] The worldwide population of people with insulin-deficient diabetes is increasing at a rate of 3−5% annually, with the highest increases in prevalence occurring in warm regions such as Africa, the Western Pacific, and the Middle East.[1,2] Unfortunately, insulin is prone to irreversible aggregation when exposed to high temperatures and/or agitation and requires careful storage and refrigerated transport (the cold chain) to retain activity over its shelf life. Maintaining insulin integrity presents a challenge for the pharmaceutical industry, healthcare providers, and people with diabetes worldwide.[3,4] Indeed, annual global costs for the refrigerated transport of all biopharmaceuticals globally exceed $15 billion (USD), and losses due to interruptions in the cold chain reach $35 billion (USD) annually.[4]

A primary driver of the loss of formulation integrity is the propensity of proteins to aggregate at hydrophobic interfaces when exposed to elevated temperatures.[5] In the case of insulin formulations, agitation and elevated temperatures—conditions common to worldwide transport and cold chain interruptions—increase interactions between partially unfolded insulin monomers adsorbed to interfaces, leading to nucleation of insulin amyloid fibrils.[6−9]

Recent studies in insulin stabilization have relied on covalent or noncovalent attachment of hydrophilic polymers directly to insulin or through encapsulation of insulin or insulin crystals in hydrogels.[10−15] While these strategies have successfully shielded insulin from interfacial adsorption or insulin−insulin interactions and increased insulin stability, they can also lead to increased absorption times and longer circulation times *in vivo*. A more translatable stabilization method would utilize an inactive excipient that could be incorporated into existing formulations without altering the drug pharmacokinetics.

Amphiphilic copolymers present an alternative to polymer−protein conjugation, exploiting their propensity to gather at the air−water interface to hinder insulin−interface interactions.[6,16−18] Poloxamers have been successfully used to improve insulin stability and have been employed in commercial formulations (Insuman U400, Sanofi-Aventis). Yet, poloxamers have not been widely adopted and have not succeeded in reducing reliance on the cold chain for insulin transport.

Previously, we have shown that biocompatible and nontoxic acrylamide carrier/dopant copolymers (AC/DC), a class of amphiphilic copolymers comprising water-soluble "carrier" and hydrophobic "dopant" monomers, can enable the development of ultrafast insulin formulations.[19] We hypothesized that these excipients could be applied more broadly to improve the stability of current commercial insulin formulations in the context of reducing cold chain reliance and improving formulation resilience. In this work, we aim to understand the stabilization mechanism of AC/DC copolymer excipients and to test the limits of their stabilizing capacity long-term and under extreme environmental conditions. We report the ability of select copolymers to act as simple "drop-in" excipients to stabilize commercial insulin formulations (Humulin R, Eli Lilly) without altering their bioactivity, pharmacokinetics, or

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pharmacodynamics, constituting an important step toward improving global access to these critical drugs.

EXPERIMENTAL METHODS

Materials. Humulin R (Eli Lilly) was purchased and used as received. Solvents *N*,*N*-dimethylformamide (DMF; HPLC Grade, Alfa Aesar, >99.7%), hexanes (Fisher, certified ACS, >99.9%), ether (Sigma, Certified ACS, anhydrous, >99%), and $CDCl_3$ (Acros, >99.8%) were used as received. Monomers *N*-(3-methoxypropyl)-acrylamide (MPAM; Sigma, 95%) and 4-acryloylmorpholine (MORPH; Sigma, >97%) were filtered with basic alumina prior to use. Monomers *N*-phenylacrylamide (PHE; Sigma, 99%) and *N*-isopropylacrylamide (NIPAM; Sigma, >99%) were used as received. Reversible addition−fragmentation *chain*-transfer agents 2-cyano-2-propyl dodecyl trithiocarbonate (2-CPDT; Strem Chemicals, >97%) and 4-((((2-carboxyethyl)thio)carbonothioyl)thio)-4-cyanopentanoic acid (BM1433; Boron Molecular, >95%) were used as received. The initiator 2,2′-azobis(2-methyl-propionitrile) (AIBN; Sigma, >98%) was recrystallized from methanol (MeOH; Fisher, HPLC Grade, >99.9%) and dried under vacuum before use. Z-group-removing agents lauroyl peroxide (LPO; Sigma, 97%) and hydrogen peroxide (H2O2; Sigma, 30%) were used as received. Streptozotocin (99.58%) was purchased from MedChem Express. All other reagents were purchased from Sigma-Aldrich unless otherwise specified.

Surface Tension. Time-resolved surface tension of the air−solution interface was measured with a platinum/iridium Wilhelmy plate connected to an electrobalance (KSV Nima, Finland). The Wilhelmy plate was partially immersed in the aqueous solution in a Petri dish, and the surface tension of the interface was recorded for 50 min from the formation of a fresh interface. Equilibrium surface tension values (t = 50 min) were reported as these values more closely describe the environment in a stored vial before agitation. Two replicates were taken and averaged.

Interfacial Rheology. Interfacial shear rheology was measured using a Discovery HR-3 rheometer (TA Instruments) with an interfacial geometry comprising a Du Noüy ring made of platinum/iridium wires (CSC Scientific, Fairfax, VA, catalog no. 70542000). Before each experiment, the Du Noüy ring was rinsed with ethanol and water and flame treated to remove organic contaminants. The solution chamber consisted of a double-wall Couette flow cell with an internal Teflon cylinder and an external glass beaker. A time sweep was performed with a strain of 1% (within the linear regime) and a frequency of 0.05 Hz (low enough for instrument inertia to not be significant). Interfacial complex shear viscosity was measured for 30 min. The experiment was repeated in triplicate.

Polymer Synthesis. Polymers were synthesized *via* reversible addition fragmentation transfer, as described in the previous literature. Detailed methods can be found in the Supporting Information. The resulting composition and molecular weights were determined *via* ^{1}H NMR spectroscopy and size exclusion chromatography (SEC) with poly(ethylene glycol) standards (Table S1).

***In Vitro* Insulin Stability Assay (Accelerated Aging).** A total of 50 μL of the AC/DC excipient ($MoNi_{23\%}$, $MpPhe_{8\%}$, $MoPhe_{6\%}$) in Milli-Q water (2.1, 21, or 105 mg/mL) or 50 μL of Milli-Q water was added to 1 mL of Humulin R (Eli Lilly 100U) in a glass autosampler vial (J.G. Finneran, 2.0 mL Clear R.A.M. large opening vial, 12 × 32 mm, 9 mm thread) and capped, yielding 95U Humulin either as a control or formulated with 0.01, 0.1, or 0.5 wt % AC/DC excipient. These vials were incubated at 37 °C and agitated at 150 rpm for 2, 4, and 6 months (in addition, Humulin-only control was agitated at 2 weeks and 1 month). The preparation of formulations was staggered so that all samples reached their end-point age at the same time. Vials were refrigerated until testing upon reaching the selected aging time point. Following initial transmittance experiments, all further experiments were done with formulations with 0.01 wt % AC/DC excipient to minimize copolymer concentration. In addition, 500 μL of 2.1 mg/mL $MoNi_{23\%}$ or Milli-Q water was added to 10 mL of unadulterated Humulin (Eli Lilly 100U) in its commercial vial to generate 95U Humulin control and formulation with 0.01 wt % AC/DC excipient. These vials were placed in the original individual packaging boxes with the instruction papers. These packages were incubated at either 37 or 50 °C until significant opacity change; 300−400 μL aliquots were removed every 24 h for the first 7 days and refrigerated. Following that, intermittent aliquots were taken to conserve volume. Every 24 h, the bottoms of the vials were photographed to track the change in opacity. Methods for aggregation assays for recombinant human insulin were adapted from Webber *et al.*[11] Formulation samples were plated at 150 μL per well in a clear 96-well plate, and an absorbance reading was taken at 540 nm (BioTek Synergy H1 microplate reader). The aggregation of insulin leads to light scattering, which results in an increase in the measured absorbance. The time to aggregation (t_A) was defined as the time point when a 10% increase in transmittance from time zero was observed.

Circular Dichroism. Circular dichroism was used to validate that aging with AC/DC excipients does not result in changes to the secondary structure of insulin. Aged Humulin (0.5, 1, 2, 4, and 6 months) or Humulin aged with 0.01 wt % AC/DC excipients (2, 4, and 6 months) was evaluated against an unaged Humulin control or unaged Humulin with 0.01 wt % AC/DC excipient. Formulation samples were diluted to 0.2 mg/mL in PBS (pH = 7.4). The samples were left to equilibrate for 15 min at room temperature before measurement. Near−UV circular dichroism spectroscopy was performed at 20 °C with a J-815 CD spectropolarimeter (Jasco Corporation) over a wavelength range of 200−260 nm using a 0.1 cm path-length cell.

***In Vitro* Insulin Cellular Activity Assay.** *In vitro* insulin activity was tested using the AKT phosphorylation pathway using AlphaLISA SureFire Ultra (PerkinElmer) kits for detection of phosphorylated AKT 1/2/3 (pS473) compared to total Akt1. Humulin, Aged Humulin (t = 6 months), Humulin + $MoNi_{23\%}$, and Aged Humulin + $MoNi_{23\%}$ (t = 6 months) formulations were tested. Methods have been previously described elsewhere.[13,14] Detailed methods can be found in the Supporting Information. Results were plotted as a ratio of [pAKT]/[AKT] for each sample (n = 3 cellular replicates) and an EC50 regression [log(agonist) *vs.* response (three parameters)] was plotted using GraphPad Prism 8.

***In Vitro* Cytotoxicity of AC/DC Excipients.** NIH 3T3 culture: NIH 3T3s were cultured according to ATCC's recommendations. Media consisted of Dulbecco's modified Eagle medium, 10% fetal bovine serum and 1% penicillin−streptomycin. The cells were split at an approximate ratio of 1:4 every 3−4 days. The Promega CellTiter-Glo 3D cell viability assay was used to characterize the short-term cell viability under different formulation conditions. The cells were seeded at 10,000 cells per well in an opaque 96-well plate in 100 μL of media containing polymers of interest. $MoNi_{23\%}$, $MPPhe_{8\%}$, and $MoPhe_{6\%}$ polymers were added to media at concentrations of 10, 5, 2.5, 1, 0.5, and 0.1 mg/mL. Four wells were used as replicates for each polymer concentration. Relative viability was measured after 1 day in culture by adding 100 μL per well of the CellTiter-Glo reagent, mixing for 5 min, allowing the plate to sit for 25 min, and then reading the luminescent signal with a 1 s integration time. LC_{50} values were determined for each polymer by plotting the absolute signal for each of the four replicates in GraphPad Prism 9 and using the [Agonist] *vs.* response−Find ECanything nonlinear fit. Fit parameter F was constrained to 50, the bottom was constrained to 4 (the negative control for the assay), and the top was constrained to be the same for all data sets (cell viability should be equal for all data sets as the polymer concentration approaches 0).

Streptozotocin-Induced Model of Diabetes in Rats. Male Sprague Dawley rats (Charles River) were used for experiments. Animal studies were performed in accordance with the guidelines for the care and use of laboratory animals; all protocols were approved by the Stanford Institutional Animal Care and Use Committee. The protocol used for streptozotocin induction was adapted from the protocol by Wu and Huan[20] and has been previously described.[13,21] Detailed methods are included in the Supporting Information. Diabetes was defined as having three consecutive blood glucose measurements >300 mg/dL in nonfasted rats.

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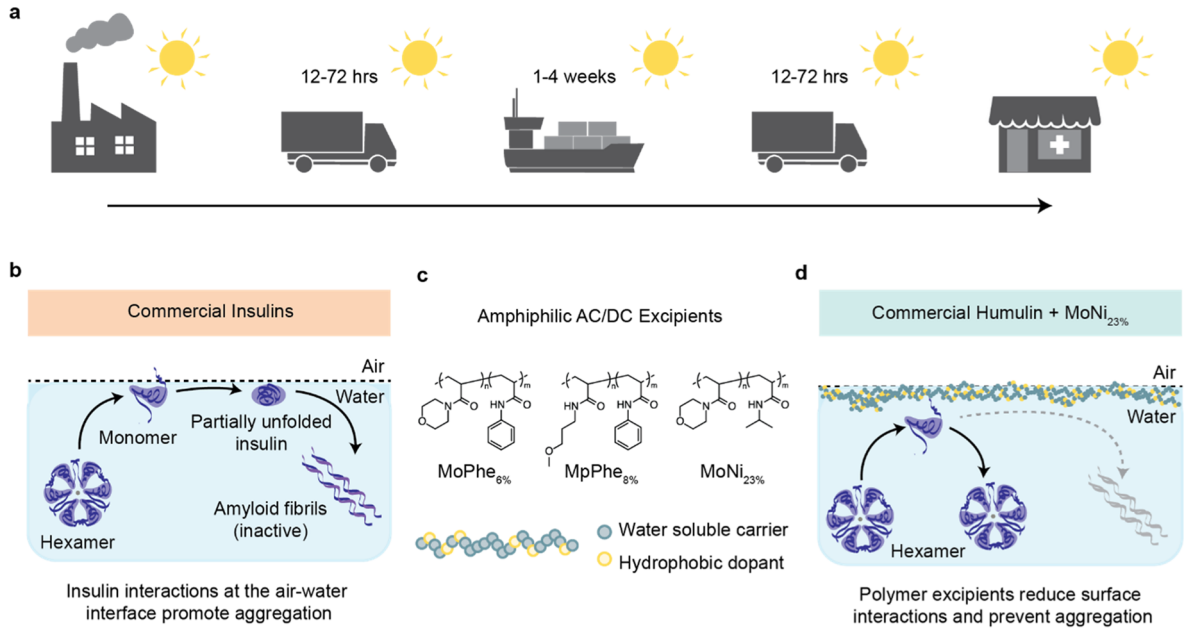

Figure 1. Scheme of the cold chain and insulin aggregation mechanism. (a) To maintain integrity, commercial insulin formulations must currently be transported and stored in refrigerated containers for the week-long duration of worldwide distribution. (b) Aggregation mechanism of commercial insulin formulations. The insulin hexamer is at equilibrium with monomers in formulation. These monomers interact at the interface, where the exposure of hydrophobic domains during insulin−insulin interaction nucleate amyloid fiber formation. (c) Chemical structure of AC/DC excipients, poly(acryloylmorpholine$_{77\%}$-*co*-*N*-isopropylacrylamide$_{23\%}$) (MoNi$_{23\%}$). AC/DC excipients have a molecular weight between 2−5 kDa (See Table S1). (d) AC/DC excipients are amphiphilic copolymers that adsorb to interfaces, reducing insulin−insulin interactions and delaying the nucleation of insulin amyloidosis.

***In Vivo* Pharmacodynamics in Diabetic Rats.** Diabetic rats were fasted for 4−6 h. For initial blood glucose studies, rats were injected subcutaneously (1.5 U/kg) with the following formulations: (i) Humulin or (ii) Humulin with 0.01 wt %AC/DC excipient (MoNi$_{23\%}$, MpPhe$_{8\%}$, and MoPhe$_{6\%}$). Humulin formulations were tested at six aging time points of 0, 0.5, 1, 2, 4, and 6 months and Humulin with AC/DC excipient was tested at 0, 2, 4, and 6 months of aging. The preparation of formulations was staggered so that all samples reached their end-point age at the same time and all aging time points could be compared in the same cohort of rats. A total of 32 rats with fasting glucose levels >300 mg/dL were randomized to a formulation group (8 rats/group), and each rat received that formulation at all levels of aging (the order of the aging time points rats received was also randomized). For blood glucose studies after formulation aging at 50 °C, rats were injected subcutaneously (1.5 U/kg) with the following formulations: (i) Humulin, (ii) aged Humulin (t = 1 day), (iii) Humulin + MoNi$_{23\%}$, or (iv) aged Humulin + MoNi$_{23\%}$ (t = 4 days). A total of 16 rats with fasting glucose levels >300 mg/dL were randomized to either the Humulin control group or the MoNi$_{23\%}$ group. Within both groups, the order that the aged formulations were given was also randomized and formulations were administered on separate experimental days. Before injection, baseline blood glucose was measured. After injection, blood was sampled every 30 min for 5 h. Blood glucose was measured using a handheld blood glucose monitor. The maximum change in blood glucose measured from the baseline was used as a metric of bioactivity of each formulation to assess *in vivo* bioactivity after aging.

***In Vivo* Pharmacokinetics in Diabetic Rats.** The diabetic rats were fasted for 4−6 h. For pharmacokinetic studies, the rats were injected subcutaneously (1.5 U/kg) with the following formulations: (i) Humulin, (ii) aged Humulin (t = 6 months), (iii) Humulin + MoNi$_{23\%}$, or (iv) aged Humulin + MoNi$_{23\%}$ (t = 6 months). A total of 16 diabetic rats were randomized to a formulation group: Humulin or Humulin + MoNi$_{23\%}$ (8 rats/group). Within each group, the rats received both the fresh (t = 0 months) or aged (t = 6 months) formulations in a randomized order. After subcutaneous injection, blood was sampled every 15 min for 2 h and blood was collected in serum tubes (Sarstedt) for analysis with ELISA. Serum insulin concentrations were quantified using a human insulin ELISA kit (Mercodia).

Statistics. All data are shown as mean ± standard error unless specified. For the *in vitro* activity assay (AKT), EC$_{50}$ regression [log(agonist) *vs.* response (three parameters)] was plotted using GraphPad Prism 8. GraphPad Prism 8 Extra sum-of-squares F-test was used to test if log(EC$_{50}$) differed between the data sets. The data sets were compared in pairs, and Bonferroni posthoc tests were used to adjust for multiple comparisons (α = 0.008). For blood glucose measurements, a restricted maximum likelihood (REML) repeated-measure mixed model was used to test for differences at different aging time points within a formulation (JMP Pro 14). The rat was included as a random effect and the age of the formulation as a within-subject fixed effect. A posthoc Tukey Honest Significant Difference (HSD) test was used on Humulin formulations to determine statistical significance between aging time points.

RESULTS AND DISCUSSION

AC/DC Excipient Insulin Stabilizing Mechanism. We selected our three top-performing candidates from a previous screen of amphiphilic AC/DC excipients for their ability to stabilize monomeric insulin.[19] These excipients were composed of either acryloylmorpholine (Mo) or methoxypropylacrylamide (Mp) as a hydrophilic carrier monomer copolymerized with either *N*-isopropylacrylamide (Ni) or phenylacrylamide (Phe) as a hydrophobic dopant monomer. This excipient design is hypothesized to preferentially occupy the air−water interface and consequently inhibit insulin−insulin interactions occurring at these interfaces (Figure 1). We sought to evaluate this hypothesis through time-resolved surface tension and interfacial rheology experiments with a model AC/DC excipient, poly(acryloylmorpholine$_{77\%}$-*co*-*N*-Isopropylacrylamide$_{23\%}$) (MoNi$_{23\%}$), coformulated with commercial Humulin R (Eli Lilly) (Figure 2).

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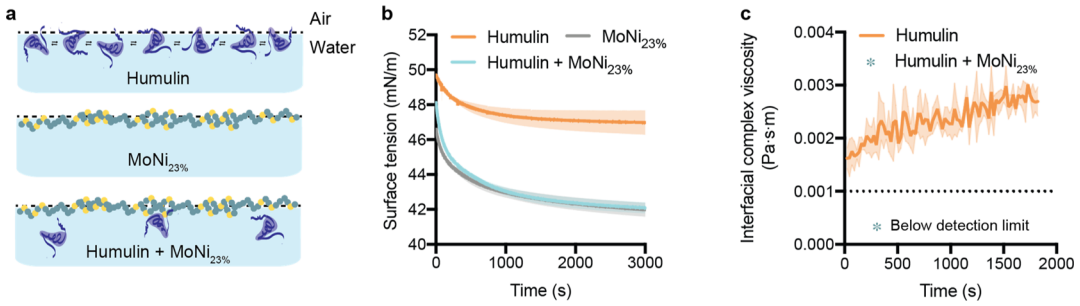

Figure 2. Experimental insights into the mechanism of AC/DC excipient stabilization. (a) Illustration of proposed stabilization mechanism: (i) in commercial Humulin, monomers at the interface have associative interactions. (ii) Alone, $MoNi_{23\%}$ occupies the interface without the presence of insulin. (iii) In combination with Humulin formulations, $MoNi_{23\%}$ disrupts insulin−insulin surface interactions, providing a mechanism for inhibiting aggregation. (b) Surface tension measurements of Humulin, $MoNi_{23\%}$ (0.01 wt %) formulated with formulation excipients, and Humulin formulated with $MoNi_{23\%}$ (0.01 wt %) (n = 2). (c) Interfacial rheology measurements of Humulin. Measurements for Humulin formulated with $MoNi_{23\%}$ (0.01 wt %) fell below the resolution of the instrument, indicating that there is no protein aggregation at the interface (n = 3).

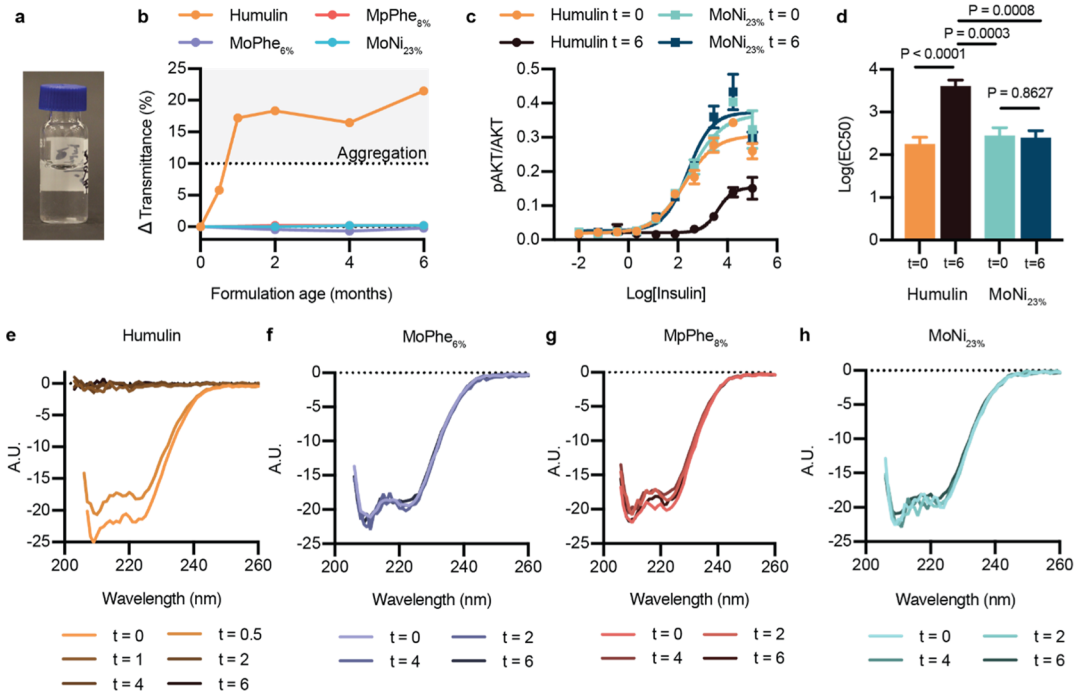

Figure 3. Formulation with AC/DC copolymers stabilizes insulin. (a) 1 mL of commercial Humulin or Humulin with the addition of AC/DC excipients: (i) $MoPhe_{6\%}$, (ii) $MpPhe_{8\%}$, (iii) $MoNi_{23\%}$ were aliquoted into 2 mL glass vials and aged at 37 °C with constant agitation (150 rpm) for 0, 2, 4, and 6 months. Additional 2-week and 1-month timepoints were added for the Humulin control. All formulations were at a concentration of 95 U/mL (diluted so that copolymers could be added to commercial Humulin). (b) Transmittance assay to assess the aggregation of proteins in formulation over time by monitoring changes in transmittance at 540 nm (n = 1 per formulation timepoint). (c) *In vitro* activity by assaying for phosphorylation of Ser^{473} on AKT after stimulation with either Humulin or $MoNi_{23\%}$ at 0- and 6-month time points. Insulin concentrations are shown as log(ng/mL). (d) Log(EC_{50}) values for each formulation. Statistical significance was assessed using the extra sum-of-squares F-test to determine if log(EC_{50}) differed between data sets. Data sets were compared in pairs, and Bonferroni posthoc tests were used to adjust for multiple comparisons (α = 0.008). (e−h) Circular dichroism spectra from 200−260 nm for each formulation (diluted to 0.2 mg/mL in PBS) at each time point. (c) Results shown are mean ± SE plotted as a ratio of [pAKT]/[AKT] for each sample (n = 3 cellular replicates) and an EC_{50} regression [log(agonist) *vs.* response (three parameters)] was plotted using GraphPad Prism 8. (d) Statistical significance was assessed using the extra sum-of-squares F-test to determine if log(EC_{50}) differed between datasets. Data sets were compared in pairs, and Bonferroni posthoc tests were used to adjust for multiple comparisons (α = 0.008).

Equilibrium surface tension measurements of Humulin R, Humulin R containing $MoNi_{23\%}$ (0.01 wt %), and a solution of $MoNi_{23\%}$ (0.01 wt %) containing the same formulation excipients (*i.e.*, Humulin R without the insulin) revealed that the presence of $MoNi_{23\%}$ resulted in surface tension values well below Humulin R (approximately 42 *vs.* 47 mN/m, Figure 2b). Moreover, a 10-fold increase in the $MoNi_{23\%}$ concentration (0.1 wt %) further reduced the surface tension of the formulation (Supplementary Figure 1A). The decrease in surface tension upon addition of $MoNi_{23\%}$ to Humulin indicates that there are more species at the interface when $MoNi_{23\%}$ and Humulin are formulated together, compared to Humulin alone. The decreased surface tension concomitant with the increased concentration of $MoNi_{23\%}$ in the absence of Humulin indicates that the surface is not saturated at 0.01 wt % $MoNi_{23\%}$. However, the surface tension is identical for

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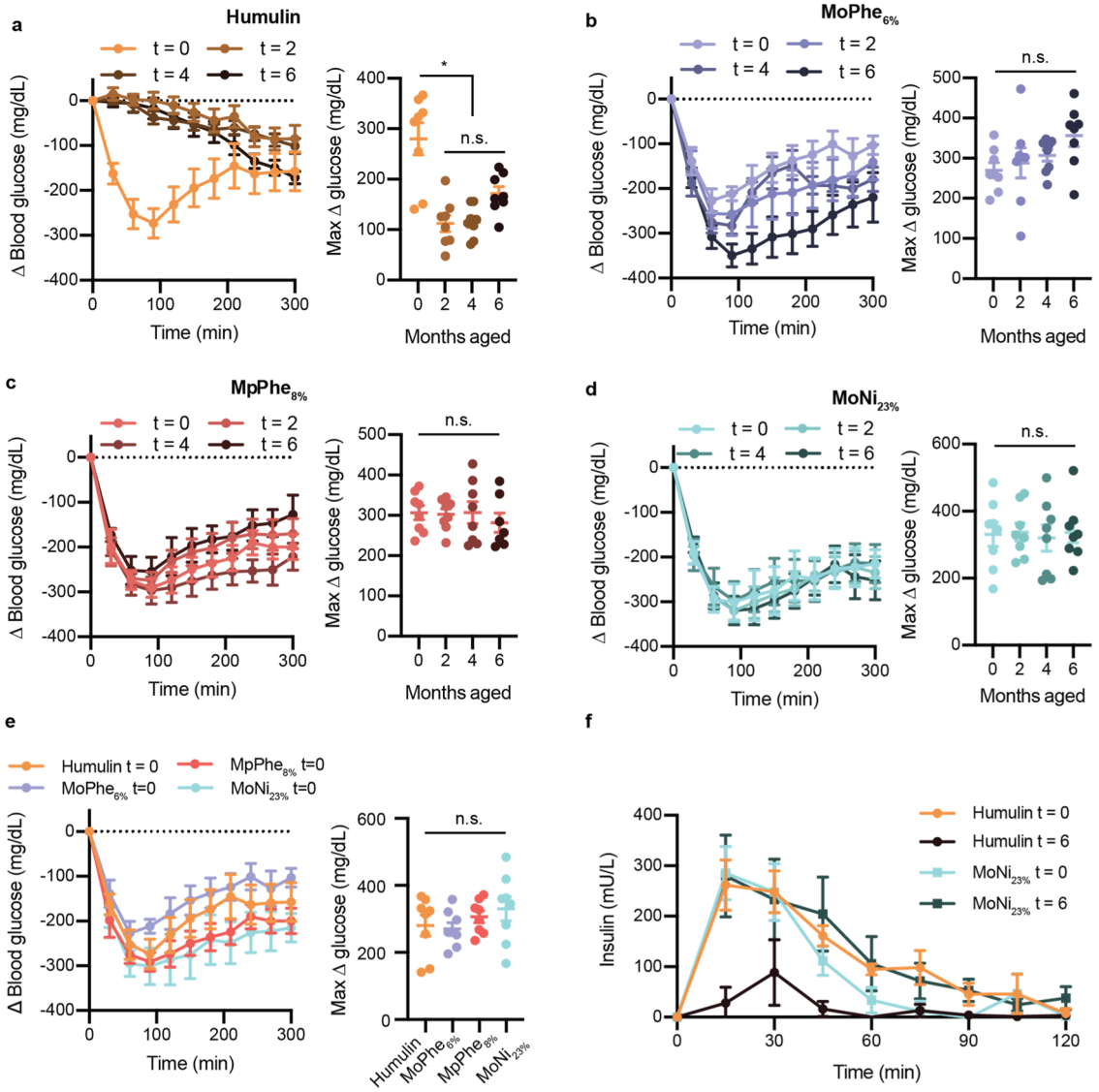

Figure 4. Insulin activity after aging in diabetic rats. Fasted diabetic male rats received subcutaneous administration (1.5 U/kg) of each insulin formulation: (a) Humulin, (b) Humulin with $MoPhe_{6\%}$, (c) Humulin with $MpPhe_{8\%}$, or (d) Humulin with $MoNi_{23\%}$ at each aging time point (0, 2, 4, and 6 months). (e) Comparison of each formulation a t = 0 months. In these assays, 32 rats were randomly assigned to one of the four formulation groups (n = 8) and each rat received one dose of the formulation at each aging time point in a random order. Blood glucose levels were measured every 30 min using a handheld glucose monitor, and the change in blood glucose relative to baseline glucose measurements was plotted. Baseline glucose measurements ranged from 300−600 mg/dL (see Supporting Information Figure S5 for raw glucose curves). The maximum difference in glucose from baseline (Δ glucose) was also plotted for each formulation as a measure of formulation potency. (f) Pharmacokinetics of Humulin and Humulin with $MoNi_{23\%}$ at t = 0 and t = 6 months. All data are shown as mean ± SE. Statistical significance between max Δ glucose was assessed using a REML repeated-measure mixed model with the rat as a random effect and the age of the formulation as a within-subject fixed effect. A posthoc Tukey HSD test was used on Humulin formulations to determine statistical significance between aging time points.

formulations of Humulin and $MoNi_{23\%}$ and $MoNi_{23\%}$ with formulation excipients, indicating that there are a similar number of molecular species at the interface regardless of the presence of Humulin. Further, the addition of completely hydrophilic poly(acryloylmorpholine) (Mo) to Humulin did not lower the surface tension, indicating that the amphiphilic copolymer is required to displace insulin (Supporting Information Figure S1B). Together, these surface tension experiments help demonstrate that $MoNi_{23\%}$ preferentially adsorbs and dominates the air−water interface.[22]

Interfacial shear rheology measurements demonstrated that addition of $MoNi_{23\%}$ (0.01 wt %) to Humulin R reduced interfacial complex viscosity to below the detection limit of the instrument compared to Humulin R, which exhibited values between 0.002−0.003 Pa·s·m (Figure 2c). The complex viscosity of Humulin is indicative of associative insulin−insulin interactions that can dissipate viscous energy at the interface. While not quantitative, the lowering of the interfacial complex viscosity below instrument detection limits is indicative that the addition of $MoNi_{23\%}$ disrupts insulin−insulin interactions at the interface.

When this complex interface is subjected to interfacial stresses and agitation, it is likely that these insulin−insulin associations can nucleate amyloid fibril formation and lead to aggregation. Together, the surface tension and interfacial rheology experiments suggest a mechanism of AC/DC enhanced insulin stabilization, where preferential adsorption

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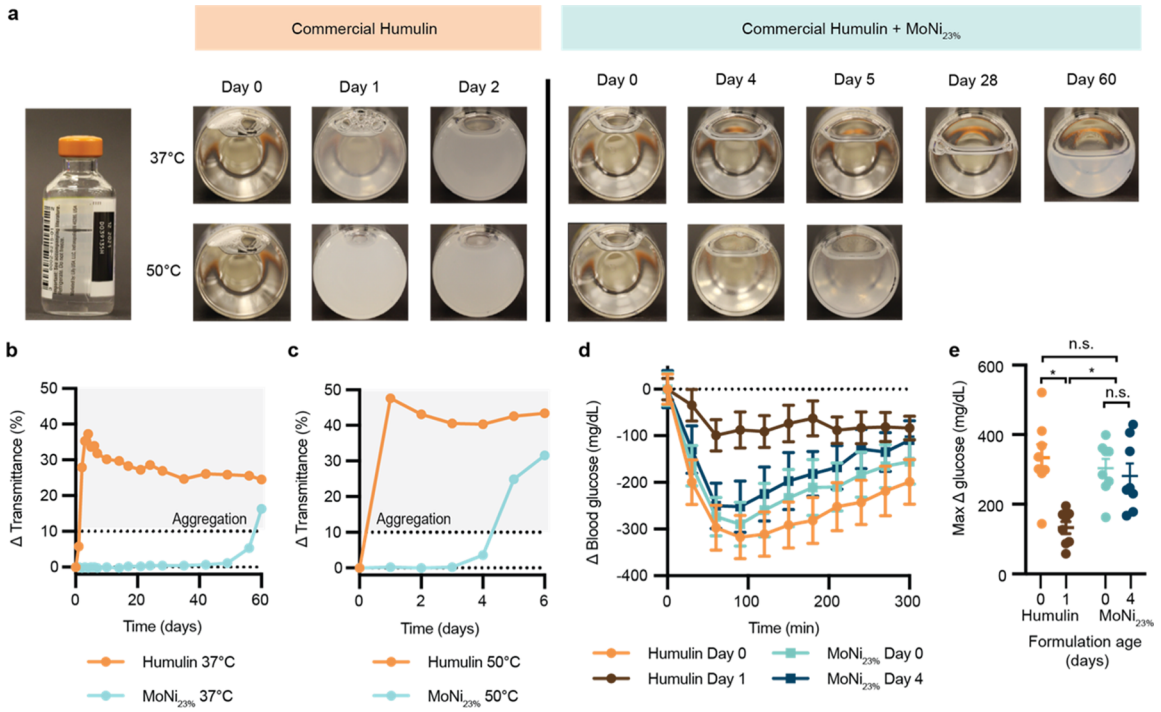

Figure 5. Stressed aging in commercial packaging. (a) Humulin is often sold in standardized 10 mL glass vials and packaged in cardboard boxes. Here, we tested the stabilizing capacity of AC/DC copolymers in commercial packaging conditions under stressed conditions. 10 mL vials of U100 Humulin R were diluted to 95 U/mL with the addition of 50 μL of a $MoNi_{23\%}$ stock solution (to a final concentration of 0.01 wt % copolymer) or water (control). Dilution was necessary to add copolymers to the formulation. These vials were replaced in their original boxes and taped to a shaker plate (150 rpm) in a 37 °C (n = 1 per formulation) or 50 °C (n = 1 per formulation) incubator. Samples were observed and imaged daily. (b,c), Transmittance assays for Humulin or Humulin comprising $MoNi_{23\%}$ after aging at (b) 37 °C and (c) 50 °C. Single samples (n = 1) were tested for each transmittance curve. (d) Blood glucose curves and (e) maximum change in blood glucose (Δ glucose) in fasted diabetic rats for samples aged at 50 °C. Data are shown as mean ± SE. Statistical significance between max Δ glucose was assessed using a REML repeated-measure mixed model with rats as a random effect and the age of the formulation as a within-subject fixed effect. A posthoc Tukey HSD test was used to determine statistical significance between aging time points and groups.

of the AC/DC excipient to the air−water interface disrupts insulin−insulin interactions.

AC/DC Excipients for Long-Term Stability of Insulin. We then sought to evaluate the capacity of our three previous top-performing AC/DC excipients, $MoNi_{23\%}$, poly(acryloylmorpholine$_{94\%}$-*co*-phenylacrylamide$_{6\%}$) ($MoPhe_{6\%}$) and poly(methoxypropylacrylamide$_{92\%}$-*co*-phenylacrylamide$_{8\%}$) ($MpPhe_{8\%}$) to act as simple "drop-in" excipients to stabilize Humulin R through stressed aging. Formulations of Humulin alone or Humulin with an AC/DC excipient added were prepared and aged for 0, 2, 4, or 6 months at 37 °C with constant agitation (150 rpm on an orbital shaker plate). The preparation of formulations was staggered so that all samples reached their end-point age at the same time. Both visual inspection and a transmittance assays were used to determine if the insulin had aggregated (Figure 3).[11,13] Insulin aggregates scatter light, and thus, aggregation can be defined as a change in transmittance greater than 10%.[11,13] Humulin alone began to aggregate after 2 weeks of stressed aging. In contrast, all insulin formulations containing AC/DC excipients $MoPhe_{6\%}$, $MpPhe_{8\%}$, and $MoNi_{23\%}$ at concentrations of 0.01, 0.1, or 0.5 wt % did not show any signs of insulin aggregation over the course of the 6-month study, with the exception of $MpPhe_{8\%}$ at 0.5 wt % (Figure 3b, Supporting Information Figure S2). Thus, to minimize the amount of copolymer excipients in the formulation, only the 0.01 wt % formulations were used for the rest of the studies reported here.

To further validate the transmittance results, which only assess insulin aggregation, we evaluated *in vitro* activity by assaying for phosphorylation of Ser^{473} on protein kinase B (AKT) after stimulating C2C12 cells with either Humulin or Humulin containing $MoNi_{23\%}$ (0.01 wt %) at both the 0- and 6-month time points (Figure 3c,d). Fresh formulations and the aged Humulin + $MoNi_{23\%}$ formulation showed equivalent bioactivity [$Humulin_{t=0}$ $\log(EC_{50})$ = 2.252 ± 0.158; $MoNi_{23\%,t=0}$ $\log(EC_{50})$ = 2.448 ± 0.186; $MoNi_{23\%,t=6}$ $\log(EC_{50})$ = 2.405 ± 0.158], whereas aged Humulin R exhibited almost complete loss of bioactivity [$Humulin_{t=6}$ $\log(EC_{50})$ = 3.606 ± 0.139] (Figure 3c,d).

While these *in vitro* AKT assay results supported the transmittance data, we sought to further confirm insulin formulation integrity by using circular dichroism to observe the insulin secondary structure for each formulation time point (Figure 3e−h). Formulations stabilized with the AC/DC excipients exhibited no changes in secondary structure after stressed aging, whereas Humulin alone had lost all structural features by 1 month. These data corroborate both the transmittance and *in vitro* activity data.

Bioactivity of Aged Insulin in Diabetic Rats. To evaluate the integrity of the aged insulin formulations in a functional setting *in vivo*, we assessed formulation activity in diabetic rats. Administration of streptozotocin was used to induce insulin-dependent diabetes in a cohort of 32 male rats. These rats were randomly assigned to one of four formulation groups: (i) Humulin or Humulin comprising either (ii)

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$MoPhe_{6\%}$, (iii) $MpPhe_{8\%}$, or (iv) $MoNi_{23\%}$ at 0.01 wt %, and each rat received that formulation at each aging time point (0, 2, 4, and 6 months). The preparation of formulations was staggered so that all samples reached their end-point age at the same time and all aging timepoints could be compared in the same cohort of rats. Insulin was administered subcutaneously in fasted rats (1.5 U/kg) and blood glucose levels were measured every 30 min. Active formulations resulted in a distinct initial drop in blood glucose from extreme hyperglycemia that reached a minimum in the range of normoglycemia between 60 and 100 min after administration (Figure 4, Supporting Information Figure S3). After this phase, blood glucose levels began to rise as insulin was cleared. In contrast, formulations that appeared aggregated in the *in vitro* transmittance assays following aging did not show this distinct reduction in glucose reminiscent of insulin action and instead resulted in a gradual decrease in glucose levels. The gradual decrease in glucose may suggest that some of the insulin is initially trapped in reversible aggregates, and over time, these aggregates dissociate and result in a slow-acting insulin effect. The maximum difference in blood glucose from baseline to the minimum glucose levels was plotted for each formulation as a measure of formulation potency. All copolymer-stabilized formulations showed no difference in activity between aging time points, but Humulin alone demonstrated a large difference between aging time points ($F_{3,21} = 23.83$, $P < 0.0001$), where a posthoc Tukey HSD test revealed that Humulin after 2, 4, and 6 months of aging had decreased activity compared to fresh Humulin ($t = 0$ months). These observations were corroborated by evaluation of insulin pharmacokinetics, where no differences were observed between fresh Humulin R ($t = 0$ months) and Humulin + $MoNi_{23\%}$ initially ($t = 0$ months) and after 6 months of aging, but a decrease in exposure was observed for the aged Humulin ($t = 6$ months) (Figure 4f, Supporting Information Figure S4). These data suggest that AC/DC excipients function as stabilizing ingredients for commercial formulations such as Humulin R without altering the insulin pharmacokinetics or pharmacodynamics.

High-Temperature Aging of Insulin Formulations. To determine the capacity of AC/DC excipients to improve insulin cold-chain resilience, we evaluated the extent of stability imbued by one of our excipients, $MoNi_{23\%}$, under extreme manufacturing and distribution conditions (37 and 50 °C with constant agitation) (Figure 5). $MoNi_{23\%}$ was selected for further testing following cytotoxicity tests that revealed that $MoNi_{23\%}$ and $MoPhe_{6\%}$ had more favorable LC_{50} values compared to $MPPhe_{8\%}$ (Supporting Information Figure S6). These results, combined with previous success using $MoNi_{23\%}$ to stabilize monomeric insulin,[19] led us to choose to advance $MoNi_{23\%}$ through the extreme aging tests. Temperatures were selected to represent the temperature on a hot summer day (37 °C) and the upper temperature range that a shipping container or truck without refrigeration or insulation could reach during the peak of summer (50 °C).[23] Humulin R can be purchased in 10 mL glass vials that are packaged and shipped in cardboard boxes (Figure 5a, Supporting Information Figure S7). $MoNi_{23}$ (0.01 wt %) was added to new vials of Humulin R using a syringe (dilution from 100 to 95 U/mL to allow addition of copolymer; control vial was diluted with water) and the vials were then replaced in the original cardboard packaging with the package insert (Supporting Information Figure S7a). The cardboard packaging was affixed to a rotary shaker inside a temperature-controlled incubator and agitated at 150 rpm (Supporting Information Figure S7b).

Visual inspection combined with a transmittance assay was used as the primary measure of insulin integrity (Figure 5a). These assays are consistent with our earlier experiments that demonstrated that the transmittance readings correlate well with both *in vitro* and *in vivo* functional activity assays. At 37 °C, Humulin alone begins to show visual changes in opacity at day 1 and becomes fully opaque by day 2. In contrast, when formulated with $MoNi_{23\%}$, the insulin formulation shows no visual changes in opacity until day 56 and remains below a 10% change in transmittance for 56 days. At 50 °C, commercial Humulin became fully opaque within 1 day. In contrast, formulation with $MoNi_{23\%}$ extends stability under these extreme conditions to past 4 days before the formulation becomes cloudy on day 5. These qualitative observations were consistent with quantitative transmittance readings (Figure 5b,c).

To verify functional insulin activity after aging at 50 °C *in vivo*, these formulations were evaluated in diabetic rats. The ability of (i) Humulin ($t = 0$ day), (ii) aged Humulin ($t = 1$ day), (iii) Humulin with $MoNi_{23\%}$ ($t = 0$ day), or (iv) aged Humulin with $MoNi_{23\%}$ ($t = 4$ days) to decrease glucose levels was measured in fasted diabetic rats. After subcutaneous administration of formulations (1.5 U/kg), blood glucose levels were measured every 30 min (Figure 5d). Humulin ($t = 0$ day), Humulin with $MoNi_{23\%}$ ($t = 0$ day), and aged Humulin with $MoNi_{23\%}$ ($t = 4$ day) demonstrated an initial blood glucose drop that reached a minimum between 60 and 100 min after injection. These results were consistent with active formulations in earlier experiments. This characteristic glucose drop was absent in rats that received aged Humulin ($t = 1$ day), consistent with inactive formulations in earlier experiments. The maximum difference in glucose from the baseline was also plotted for each formulation as a metric of formulation potency (Figure 5e).

Statistical analysis identified a difference between the potency of these formulations ($F_{3,18.18} = 10.71$, $P = 0.0003$), whereby a posthoc Tukey HSD test revealed that aged Humulin alone had significantly decreased activity compared to the other formulations. In contrast, there was no statistical difference between unaged Humulin, unaged Humulin with $MoNi_{23\%}$, as well as aged Humulin with $MoNi_{23\%}$ after stressed aging at 50 °C for 4 days.

CONCLUSIONS

In this study, we report on the application of AC/DC excipients to imbue long-term stability and cold-chain resilience to commercial insulin formulations. While commercial insulin formulations have good shelf lives when stored properly, interruptions in the cold chain can decrease insulin bioactivity and formulation integrity. The primary mechanism of insulin destabilization is through insulin adsorption at the air−water interface, where insulin−insulin interactions promote amyloid fibril nucleation.[6,7,9] We hypothesized that amphiphilic copolymer excipients could potentially displace insulin from the interfacial region and thus improve stability by reducing the probability of nucleation of insulin−insulin aggregation events at the interface. Surface tension and interfacial rheology data on our top-performing excipient $MoNi_{23\%}$ support this hypothesis by showing that these copolymers preferentially adsorb to the air−water interface and disrupt insulin−insulin interactions. No thermoresponsive

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behavior was observed by the $MoNi_{23\%}$ excipient at the temperatures tested in this study (Figure S8); thus, disruption of surface interactions remains the most likely mechanism for stability.

When used as simple, low-concentration (0.01 wt %) formulation additives, AC/DC excipients preserve insulin activity through 6 months of stressed aging without modifying formulation pharmacokinetics, protein secondary structure, formulation clarity, or *in vivo* bioactivity. When further subjected to harsh stressed aging tests in standard packaging, Humulin R formulated with our $MoNi_{23\%}$ excipient did not aggregate for over 56 days of constant agitation at 37 °C and 4 days of constant agitation at 50 °C, whereas Humulin R alone aggregated within 2 days at 37 °C and within 1 day at 50 °C. While agitation experienced by pharmaceuticals during typical storage and transportation can be highly complex, these processes can be mimicked *in vitro* using elevated temperatures and continuous agitation. Stressed aging, through incubation at elevated temperatures with continuous agitation, has been previously used to test insulin stability.[11,13,14,19,21] Even in hot climates with limited cold chain infrastructure, it is unlikely that shipping containers would remain at 50 °C with continuous agitation with no reprieve for over 4 days and nights. Further, in our harsh stressed aging studies, we use 10 mL vials, which resulted in a higher rate of interfacial exchange compared to initial stability studies in autosampler vials due to a larger diameter. The increased stability observed for Humulin R formulated with our $MoNi_{23\%}$ excipient compared to Humulin R alone suggests that our $MoNi_{23\%}$ excipient has utility in stabilizing insulin under various agitation conditions where interfacial turnover may be higher (*i.e.*, horizontal agitation). The conditions evaluated in this study represent extreme exposure conditions during shipping in uninsulated containers or trucks in the hottest climates in the world, where transport can take weeks before reaching patients.[18] In addition to refrigerated transport in the early stages of the cold chain, maintaining proper transport and storage conditions during local distribution and once in patients' hands presents a challenge in many parts of the world.[24] This work suggests that the addition of AC/DC excipients can preserve insulin formulation integrity during even severe cold chain interruptions, enabling a reduction in cold chain requirements for insulin transportation and storage that are difficult to maintain in under resourced environments.[3,24−29]

These AC/DC excipients are synthesized at molecular weights below the glomerular filtration threshold to ensure they are eliminated without tissue accumulation, and they are prepared with facile and scalable controlled radical polymerization techniques from inexpensive starting materials. Previous exploration of this class of amphiphilic copolymer showed that they do not alter blood chemistry markers for liver and kidney function following repeated administration in rats and exhibit cytotoxicity levels comparable to the phenolic preservatives used in commercial insulin formulations.[19]

Taken together, the results reported herein demonstrate the potential of AC/DC excipients as insulin formulation additives to improve cold-chain resilience, thereby expanding global access to these critical drugs for treatment of diabetes. Further, based on the identified stabilization mechanism, AC/DC excipients are promising candidates to stabilize other biopharmaceuticals, such as monoclonal antibodies, that lose bioactivity as a result of aggregation at interfaces.[22,30] Future studies will require continued evaluation of the limits of AC/DC stabilized insulin and explore the application of these excipients to other protein therapeutics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.1c00474.

Supplemental methods, surface tension polymer formulations, transmittance assays for 5 mg/mL and 1 mg/mL formulations, blood glucose curve for Humulin, area under the curve for pharmacokinetics, insulin activity after aging in diabetic rats, cytotoxicity and LC50 for AC/DC excipients, photographs of relevant materials for high-temperature aging study, DLS measurements, and molecular information for AC/DC excipients (PDF)

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C.L.M and J.L.M contributed equally. Conceptualization, C.L.M., J.L.M., E.A.A. Methodology, C.L.M., J.L.M., A.K., C.M.M., A.K.G., A.A.A.S. Investigation, C.L.M., J.L.M., A.K., C.M.M., A.K.G., B.S.O. Writing−Original Draft, C.L.M and J.L.M. Writing−Review and Editing, C.L.M., J.L.M., A.K., C.M.M., A.K.G., B.S.O., A.A.A.S., G.G.F., E.A.A. Supervision E.A.A.

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Notes

The authors declare the following competing financial interest(s): E.A.A., J.L.M., and C.L.M. are listed as inventors on a provisional patent application (63/011,928) filed by Stanford University describing the technology reported in this manuscript.

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