

# Why does life use just 20 amino-acids?



**The universe seems to love amino acids. Maybe if we found life on a different planet, it wouldn't be that different.**

Stephen Fried, Johns Hopkins chemist. Source: <https://hub.jhu.edu/2023/02/27/protein-origin-life/>

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## **(1) Ancient proteins offer new clues about origin of life on Earth**

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**(2) Early Selection of the Amino Acid Alphabet Was Adaptively Shaped by Biophysical Constraints of Foldability** 23 Feb 2023, 12 Dec 2022  
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**(3) Supplementary Information: Early selection of the amino acid alphabet was adaptively shaped by biophysical constraints of foldability** 23 Feb 2023,

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# Ancient proteins offer new clues about origin of life on Earth

By simulating early Earth conditions in the lab, researchers have found that without specific amino acids, ancient proteins would not have known how to evolve into everything alive on the planet today -- including plants, animals, and humans.

The findings, which detail how amino acids shaped the genetic code of ancient microorganisms, shed light on the mystery of how life began on Earth.

"You see the same amino acids in every organism, from humans to bacteria to archaea, and that's because all things on Earth are connected through this tree of life that has an origin, an organism that was the ancestor to all living things," said Stephen Fried, a Johns Hopkins chemist who co-led the research with scientists at Charles University in the Czech Republic. "We're describing the events that shaped why that ancestor got the amino acids that it did."

The findings are newly published in the *Journal of the American Chemical Society*.

In the lab, the researchers mimicked primordial protein synthesis of 4 billion years ago by using an alternative set of amino acids that were highly abundant before life arose on Earth.

They found ancient organic compounds integrated the amino acids best suited for protein folding into their biochemistry. In other words, life thrived on Earth not just because some amino acids were available and easy to make in ancient habitats but because some of them were especially good at helping proteins adopt specific shapes to perform crucial functions.

"Protein folding was basically allowing us to do evolution before there was even life on our planet," Fried said. "You could have evolution before you had biology, you could have natural selection for the chemicals that are useful for life even before there was DNA."

Even though the primordial Earth had hundreds of amino acids, all living things use the same 20 of these compounds. Fried calls those compounds "canonical." But science has struggled to pinpoint what's so special -- if anything -- about those 20 amino acids.

In its first billion years, Earth's atmosphere consisted of an assortment of gases like ammonia and carbon dioxide that reacted with high levels of ultraviolet radiation to concoct some of the simpler canonical amino acids. Others arrived via special delivery by meteorites, which introduced a mixed bag of ingredients that helped life on Earth complete a set of 10 "early" amino acids.

How the rest came to be is an open question that Fried's team is trying to answer with the new research, especially because those space rocks brought much more than the "modern" amino acids.

"We're trying to find out what was so special about our canonical amino acids," Fried said. "Were they selected for any particular reason?"

Scientists estimate Earth is 4.6 billion years old, and that DNA, proteins, and other molecules didn't begin to form simple organisms until 3.8 billion years ago. The new research offers new clues into the mystery of what happened during the time in between.

"To have evolution in the Darwinian sense, you need to have this whole sophisticated way of turning genetic molecules like DNA and RNA into proteins. But replicating DNA also requires proteins, so we have a chicken-and-egg problem," Fried said. "Our research shows that nature could have selected for building blocks with useful properties before Darwinian evolution."

Scientists have spotted amino acids in asteroids far from Earth, suggesting those compounds are ubiquitous in other corners of the universe. That's why Fried thinks the new research could also have implications for the possibility of finding life beyond Earth.

"The universe seems to love amino acids," Fried said. "Maybe if we found life on a different planet, it wouldn't be that different."

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## **Early selection of the amino acid alphabet was adaptively shaped by biophysical constraints of foldability**

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## ABSTRACT

Whereas modern proteins rely on a quasi-universal repertoire of 20 canonical amino acids (AAs), numerous lines of evidence suggest that ancient proteins relied on a limited alphabet of 10 'early' AAs, and that the 10 'late' AAs were products of biosynthetic pathways. However, many non-proteinogenic AAs were also prebiotically available, which begs two fundamental questions: Why do we have the current modern amino acid alphabet, and Would proteins be able to fold into globular structures as well if different amino acids comprised the genetic code? Here, we experimentally evaluated the solubility and secondary structure propensities of several prebiotically relevant amino acids in the context of synthetic combinatorial 25-mer peptide libraries. The most prebiotically abundant linear aliphatic and basic residues were incorporated along with or in place of other early amino acids to explore these alternative sequence spaces. We show that foldability was a critical factor in the selection of the canonical alphabet. Unbranched aliphatic and short-chain basic amino acids were purged from the proteinogenic alphabet despite their high prebiotic abundance because they generate polypeptides that are over-solubilized and have low packing efficiency. Surprisingly, we find that the inclusion of a short-chain basic amino acid also decreases polypeptides' secondary structure potential. Our results support the view that despite lacking basic residues, the early canonical alphabet was remarkably adaptive at supporting protein folding and explain why basic residues were only incorporated at a later stage of the alphabet evolution.

## INTRODUCTION

The role of peptides and proteins in life's emergence has historically been sidelined as they have been, at times, perceived as only relevant after the evolution of nucleotide-based polymers, the genetic code, and a translation apparatus. However, the prebiotic abundance of amino acids and their ease of condensation resulting in polymers capable of creating functional hubs along with various cofactors (such as metal ions and organic compounds) has prompted some chemists to reconsider peptides' role during life's early evolution.<sup>1-4</sup> While extant proteins are built from a sequence space spanned by the twenty canonical amino acids (cAAs) and rely on the specificity of the Central Dogma, early peptides (and peptide-like polymers) likely arose from a larger pool of prebiotically plausible monomers.

The modern protein alphabet was most likely selected during the first 10–15% of Earth history (~4.5–3.9 Ga) but the factors guiding its chemical and biological evolution remain unclear.<sup>5</sup> Some of the cAAs are thought to be “late” additions to the genetic code, enabled only (or enriched significantly) by the evolution of their biosynthetic pathways. Indeed, many of the late amino acids are considered to be scarce in the prebiotic synthesis experiments.<sup>6</sup> “The “early” amino acids (Ala, Asp, Glu, Gly, Ile, Leu, Pro, Ser, Thr and Val) were apparently available through prebiotic synthesis along with many other non-canonical amino acids (ncAAs) and their alternatives (such as beta- and gamma- amino acids, hydroxy acids or dicarboxylic acids). Based on two independent meta-analyses and many parallel studies, the canonical alphabet was therefore selected first from the large pool of prebiotically plausible structures and then supplemented with late amino acids through metabolism.<sup>7,8</sup> Why certain amino acids were selected over others (and when) has been repeatedly discussed in literature over the last few decades.<sup>5,9-12</sup> This question can be further subdivided into two separate but related questions, namely: (i) Why were the ten early cAAs selected from the prebiotic environment, and (ii) What factors drove the selection of the additional residues in the following era? Has protein evolution been successful as a consequence of the selected cAAs, or could similar protein space be formed with alternative alphabets?<sup>9</sup>

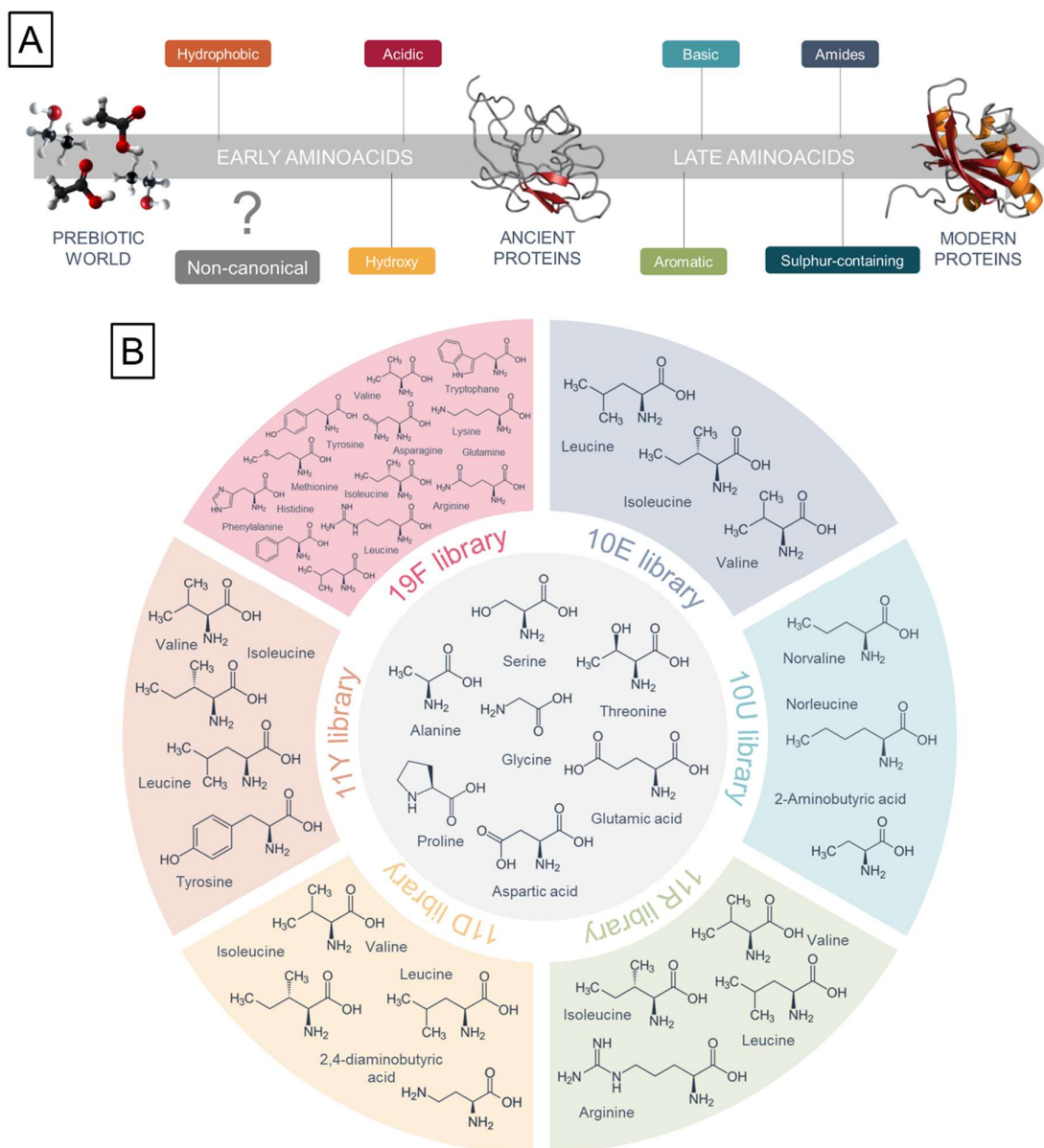
While some of the choices for the early amino acids are quite expected (especially for the C2 and C3 amino acids), others have been repeatedly considered thought-provoking. Most strikingly, the early amino acid alphabet lacked positively charged residues, even though diaminopropionic and diaminobutyric acids (C3 and C4 amino acids, DAP and DAB, respectively) have been shown to be prebiotically plausible.<sup>13,14</sup> Neither lysine nor arginine are considered prebiotically plausible, whilst ornithine is known to cyclize, promoting peptide chain scission.<sup>9</sup> Moreover, the canonical alphabet does not include some of the most abundant linear aliphatic amino acids such as  $\alpha$ -amino-n-butyric acid (ABA), norvaline (Nva) and norleucine (Nle) (C4, C5, and C6, respectively) while their branched alternatives (such as Val, Leu, Ile) were selected<sup>9</sup> despite being available at much reduced abundances. It has been hypothesized that perhaps the earlier version of the amino acid alphabet included some ncAAs that were later replaced by late cAAs.<sup>2,15</sup> This is an intriguing hypothesis especially in light of the fact that some of these ncAAs have been reported as promiscuous substrates of some aminoacyl-tRNA synthetases.<sup>16</sup>

This study experimentally determines the properties that would have accompanied some of the most feasible ncAA candidates from the prebiotic pool. We incorporated the selected ncAAs into combinatorial peptide libraries along with (or replacing) other early amino acids to evaluate their effect on fundamental physicochemical properties such as solubility and secondary structure formation. The outcomes of our study show that an inclusion of some of

the most feasible alternatives would *not* have supported the twin requirements of rich secondary structure potential and solubility. Hence, our study demonstrates that biophysical constraints on foldability provided a selective pressure shaping the building block selection in the early alphabet and therefore explains why certain early AAs (and not others) were selected to construct proteins.

## RESULTS

**Design and synthesis of peptide libraries from alternative alphabets.** 25-mer combinatorial peptide libraries were synthesized using solid-support chemical synthesis with isokinetic mixtures of amino acids (see Supplementary Table S1).



**Figure 1.** The assumed early and late stages of amino acid alphabet incorporation during protein evolution (A) and design of peptide libraries based on this order (B).

To model the sequence space available to different subsets of the amino acid alphabets, the libraries included the entire canonical alphabet without Cys (19F; F = full), its prebiotically available subset of 10 (10E; E = early), an alternative of 10E where the branched aliphatic amino acids were substituted with their unbranched prebiotically-abundant alternatives (10U; U = unbranched), the 10E library supplemented with: Arg as a representative of a modern cationic cAA (11R; R = Arg); or DAB as a representative of a potentially early cationic AA (11D; D = 2,4-diaminobutyric acid); or Tyr as a representative of an aromatic AA (11Y; Y = Tyr) (Figure 1).

MALDI spectra confirmed the expected molecular weight range and distribution of the combinatorial libraries, reflecting their respective compositions (Supplementary Figure S1). Apart from the DAB in 11D library, the amino acid analyses of all libraries confirmed the expected composition (Supplementary Figure S2). The analysis protocol could not detect DAB whose presence in the 11D library (contrary to the 10E library) was instead determined using fluorescamine assay (Supplementary Figure S3). All the libraries passed quality control and were lyophilized to establish Cl<sup>-</sup> as the counterion for all downstream analyses.

**Solubility and aggregation profiles.** The ability to remain soluble under different conditions could represent an important selection factor during the formation of the early protein alphabet, as prebiotically relevant environments spanned from alkaline hydrothermal vents to acidic lakes.<sup>17,18</sup> We measured the solubility profiles of these peptide libraries in the pH range 3–11, and also at low vs. high ionic strength, spectrophotometrically by absorption of peptide bonds at 215 nm and fluorometrically in case of library 19F due to its very poor solubility (Figure 2). In general, ionic strength did not significantly modulate solubility, with the exception of the 11Y library (see below).

At a fixed nominal peptide concentration (0.5 mg/ml), the 19F library is significantly less soluble than the libraries with narrower amino acid repertoires. This result is in line with previous studies reporting that subsets of the canonical library that are enriched with the early amino acids are significantly more soluble than the full alphabet version.<sup>19–21</sup> This phenomenon could be partly explained by the physicochemical nature of the full alphabet (such as the presence of aromatic amino acids) leading to increased aggregation propensity,<sup>22</sup> or the greater ‘search complexity’ of a larger alphabet (leading to a more rugged landscape). Regardless of the mechanism, the results support the view that peptides composed of the full alphabet were more reliant on chaperones and/or translation to fold.<sup>2,22</sup>

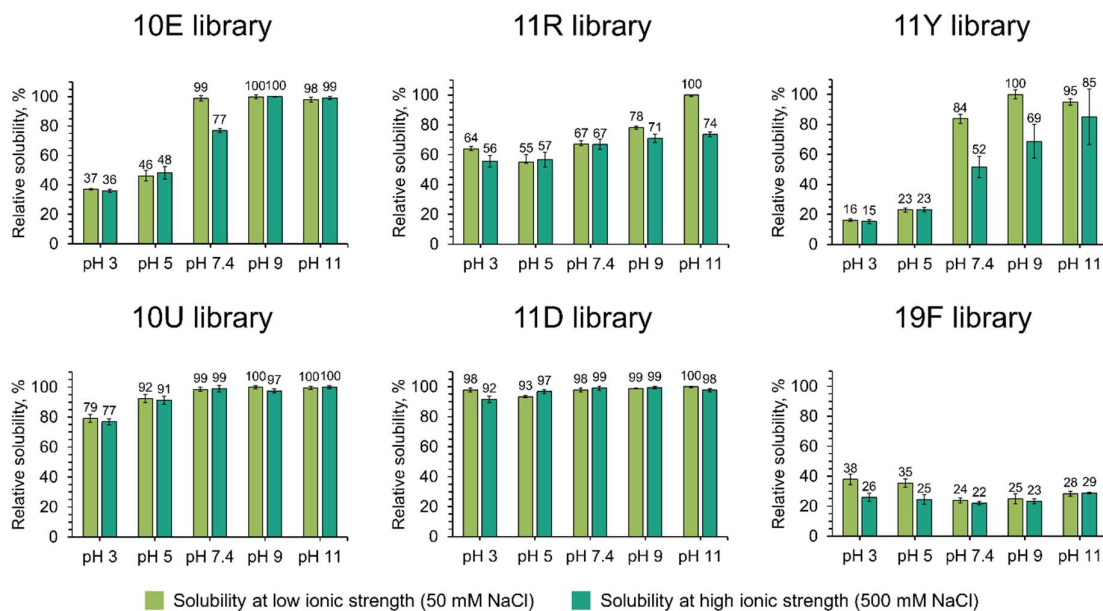
The solubilities of the canonical library subsets (10E, 11R and 11Y) trend upwards with increasing pH (going from pH 3 to pH 11) and 11Y (which includes Tyr as a representative aromatic amino acid) is the least soluble of these three as expected. This result can be potentially explained by pointing out that all of these alphabets (as do modern proteomes)<sup>23</sup> tilt toward being acidic; hence, at alkaline pH the peptides will accrue larger net negative charge and will repel one another electrostatically (inhibiting aggregation). This is broadly consistent with the practice of using mildly-alkaline buffers in protein refolding experiments, which tend to facilitate refolding by preventing aggregation.<sup>24</sup>

The two libraries that include the non-canonical alternatives of aliphatic (10U) and basic (11D) amino acids are strikingly more soluble than 10E and 11R throughout the examined pH range (Figure 2). Both of these results are unexpected for distinct reasons. Based on the physical chemistry of elementary hydrocarbons, one might expect that the 10U library would be less



soluble because linear hydrocarbons are more hydrophobic than branched hydrocarbons of equal carbon-count because they create cavities with greater surface area.<sup>25</sup> Moreover, the inclusion of a basic amino acid would raise the average isoelectric point of the library (relative to 10E) to be closer to neutral, which would decrease intermolecular repulsion, and thereby increase aggregation.

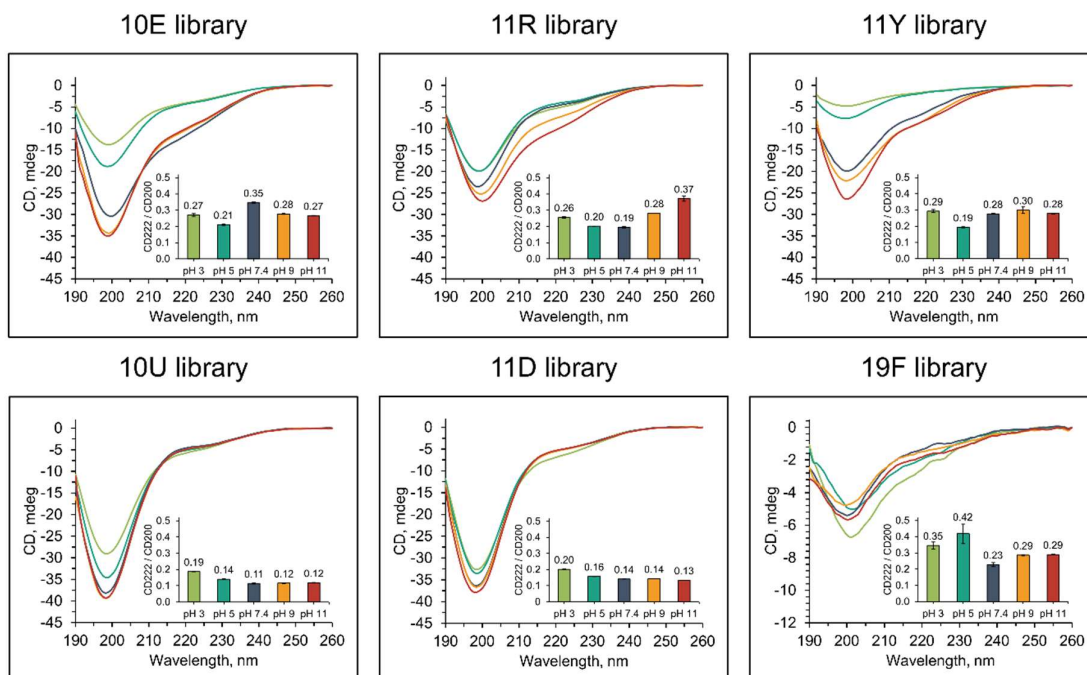
The soluble fractions of the libraries were additionally screened for the occurrence of soluble aggregates using size-exclusion chromatography (Supplementary Figures S4 and S5, in low and high ionic strength, respectively). No significant amount of such phenomenon was observed with the exception of 11Y and 10E libraries where a minor (up to ~10%) fraction of soluble aggregates was detected (Supplementary Figure S4). Overall, the elution profiles correlated with the spectrophotometric solubility measurements in which the 11R library was the most soluble of the canonical subset libraries, and the 10U and 11D libraries were fully soluble across the entire pH range.



**Figure 2.** Solubility of 25-mer combinatorial peptide libraries at different pH and ionic strengths. Solubility was measured for 0.5 mg/ml nominal peptide library solutions in 20 mM ABP buffers (pH 3–11) at 50 and 500 mM NaCl. Solubility of 19F peptide library was measured by fluorescence spectroscopy, and solubility of the other peptide libraries was measured by UV-Vis absorption spectroscopy.

**Secondary structure propensity.** Natural non-folding sequences as well as random sequences with low secondary structure content have been previously reported to be highly soluble.<sup>21,26</sup> The solubility profiles of the 11D and 10U libraries therefore suggest that the incorporation of the respective ncAAs may decrease the potential for secondary structure formation. CD spectroscopy was used to estimate average secondary structure content of the libraries over the studied pH range (Figure 3) and upon its induction with 2,2,2-trifluoroethanol (TFE) in pH 7.4 (Figure 4). Because no dominant secondary structure motif is expected in

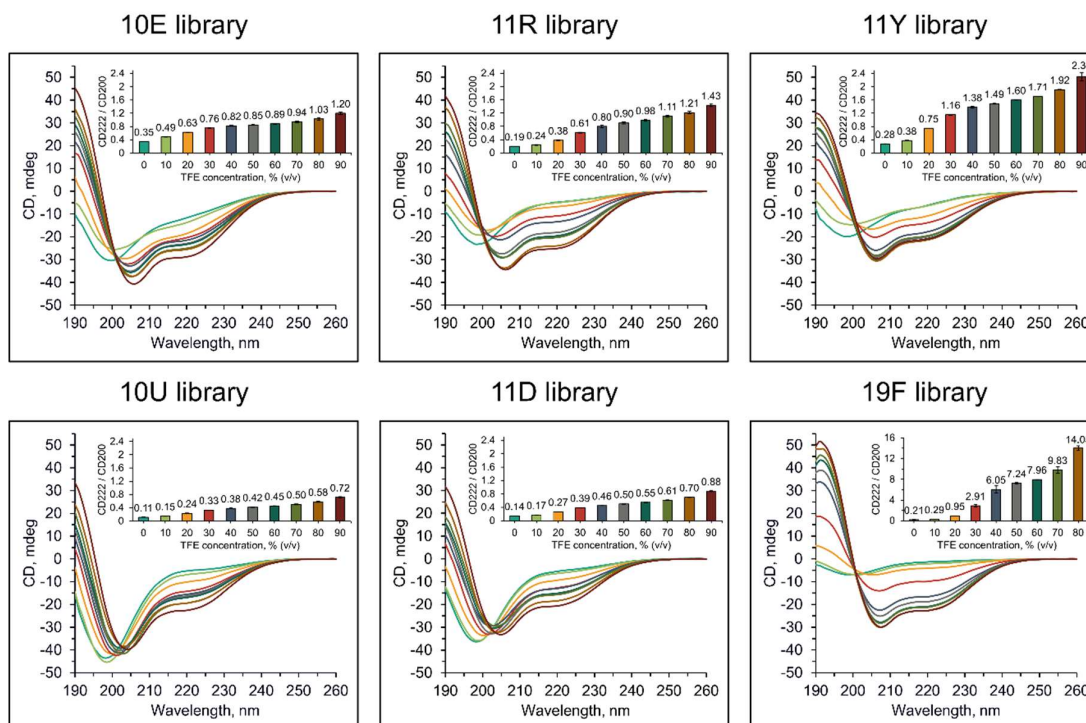
these highly diverse libraries and we are rather interested in average structural propensities, the secondary structure content was evaluated as the ratio of CD signal at 222 nm to the CD signal at 200 nm (shown by inlet graphs in Figures 3 and 4), because both alpha helices and beta sheets exhibit higher ellipticities than random coils at 222 nm.<sup>27</sup> We did not observe any significant induction of secondary structure upon changing the metal ion concentration (Supplementary Figure 6).



**Figure 3.** The effect of pH on the secondary structure of 25-mer combinatorial peptide libraries. CD spectra were recorded at a nominal concentration of 0.2 mg/ml peptide library (however is less for aggregation prone libraries, e.g. 19F) in a series of 10 mM ABP buffers (pH 3–11) at 25 mM NaCl. The inlet graph shows the ratios of CD signals at 222 nm to 200 nm.

The CD spectra of the 10U and 11D libraries showed significantly lower relative signals at 222 nm, indicating that these peptides possessed profoundly decreased secondary structural propensity when compared with the respective canonical libraries 10E and 11R (Figure 3). The 10E library with branched aliphatic amino acids has a higher structural propensity than the 10U library with unbranched aliphatic alternatives across the whole pH range. The addition of DAB in the 11D library also significantly decreases secondary structure propensity while the addition of canonical amino acids, Arg (in 11R) or Tyr (in 11Y), increases the secondary structure propensity mildly under some pH values. This trend becomes more pronounced upon TFE titration which is often used to induce helical structure.<sup>27–29</sup> TFE addition potentiates secondary structure content robustly in the 10E library, an effect that is further amplified in alphabets that include Arg and Tyr (Figure 4). Remarkably, the inclusion of DAB (in the 11D library) significantly decreases the potentiating capacity of TFE, implying that the inclusion of DAB reduces the secondary structure potential. While the poor solubility of the 19F library is

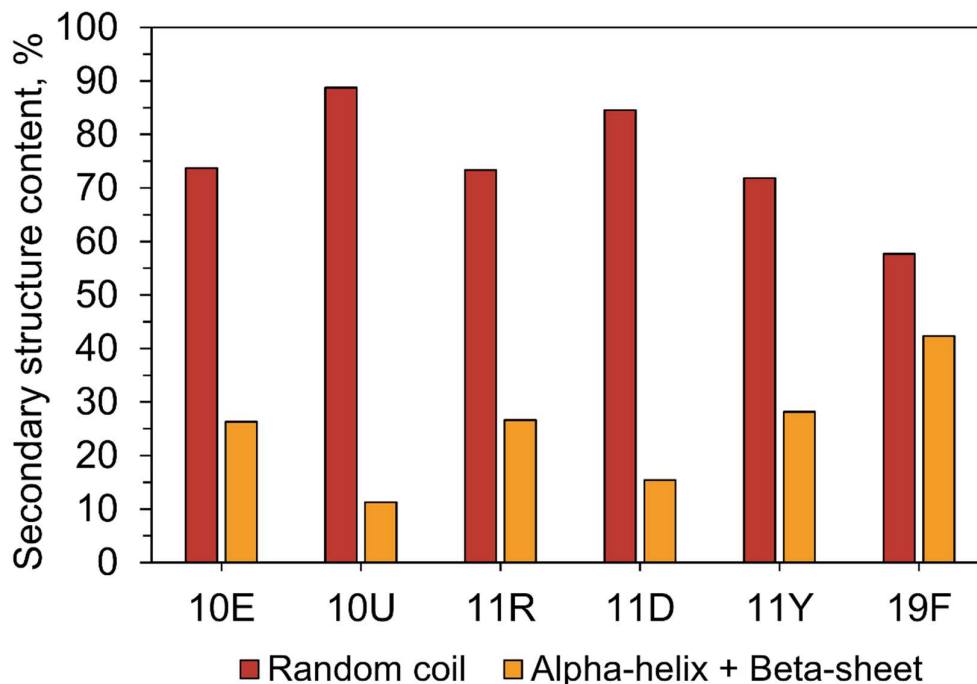
reflected in the overall low intensity of its CD spectra, its elevated structural propensity (when compared with all the other library subsets) becomes evident in the TFE titration (Figure 4).



**Figure 4.** The effect of 2,2,2-trifluoroethanol to induce secondary structure of 25-mer combinatorial peptide libraries. CD spectra were recorded at 0.2 mg/ml nominal concentration in 10 mM ABP buffer (pH 7.4) with 0–90% (v/v) 2,2,2-trifluoroethanol. The inset graphs show the ratio of CD signal at 222 to 200 nm. The y-axis has the same scale for all except for 19F library.

## DISCUSSION

The absence of ABA, Nva, and Nle in the proteinogenic alphabet has been considered striking given their high prebiotic abundance.<sup>30</sup> Some authors have commented that this universal feature of biochemistry is a potential example of a “frozen accident” due to the absence of an obvious advantage of the canonical aliphatic residues over these ncAAs. At the same time, Weber and Miller hypothesized that inclusion of linear aliphatic amino acids would increase the side chain mobility and hence would not promote formation of ordered tertiary structure.<sup>9</sup> Our experimental observations support this hypothesis. Moreover, structural prediction of 1,000 randomly chosen peptides by PEPstrMOD (using molecular dynamics based on force field libraries) confirms the same trend (Figure 5).<sup>31</sup>



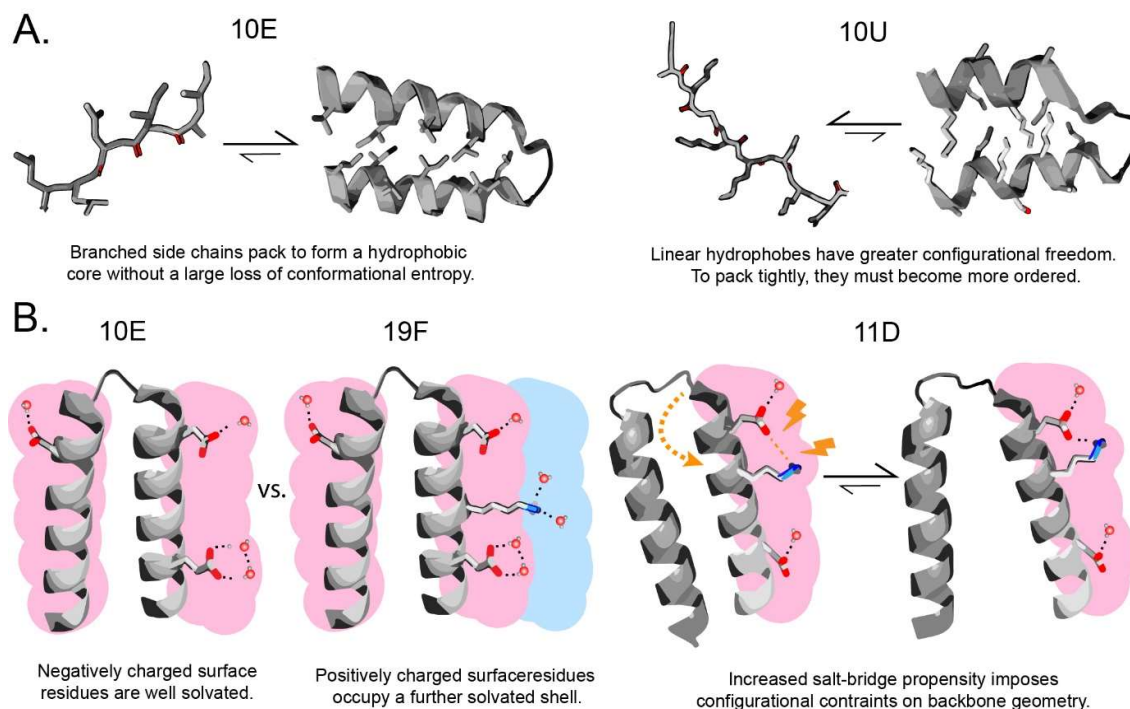
**Figure 5.** PEPstrMOD prediction of secondary structure content of 1,000 sequences selected randomly from the 25-mer combinatorial peptide libraries.

Even though unbranched amino acids are slightly more hydrophobic than their branched peers, they are expected to be much less adept at packing internal hydrophobic cores, a prerequisite to fold globular domains.<sup>32,33</sup> The model shown in Figure 6A neatly explains why the 10U library has lower secondary structure-forming potential. Specifically, unbranched hydrocarbons have more conformational freedom than their branched isomers, therefore the ordering that is implicit in packing is expected to be more entropically costly. For small peptides, the hydrogen bonding associated with secondary structure is normally isoergonic between unfolded and folded states, so the driving force for secondary structure formation arises from coupling to hydrophobic collapse.<sup>32,34–36</sup> The lower packing efficiency of these side chains simultaneously explains the high solubility of the 10U library. Hence, the requirement of foldability most likely acted to purge ABA, Nva, and Nle from the pool of amino acids that ultimately became canonical.

The more prebiotically abundant diamino acids, DAP and DAB, are also strikingly absent in the modern amino acid alphabet. One possible scenario is that DAP and DAB were utilized in an ancient alphabet, and then were ultimately displaced with Arg, Lys, and His.<sup>11</sup> Another school of thought holds that proteins lacked positively charged residues altogether until biosynthetic pathways for the prebiotically-unavailable Arg, Lys and His evolved.<sup>8,37</sup> While the second theory might seem unlikely at first glance, proteins lacking positively charged amino acids have recently been shown capable of folding and even binding with nucleic acids, assisted by metal ion cofactors.<sup>22,38–40</sup> Our results concerning the 11D library further support the second theory. Hence, this body of work is building a compelling case that primordial proteins were highly acidic.

We find that when DAB is incorporated into the 10E alphabet, it reduces secondary structure potential. Hence, its inclusion would have been a step *backward* rather than a step *forward* toward the goal of assembling foldable polypeptides. The same trend is observed in the PEPstrMOD predictions (Figure 5). The 11D library is also exceptionally soluble, so the combination of high solubility and low foldability implies that 11D polypeptides are mostly unfolded in aqueous solution.

Why does DAB harm protein structure propensity so significantly? It has been previously pointed out that these short-chain amines suffer from lactamization and acyl migration in peptides.<sup>9,41</sup> This point is important in relation to the long-term stability of peptide chains, but it cannot feasibly explain the results of our study given that lactamization is a slow reaction in relation to the timescale of our experimental work. Moreover, the MALDI spectra of the library did not show any detectable scission products. A second possibility that we propose (Figure 6B) is that DAB disrupts structural potential because of a conflict associated with its simultaneous presence with Asp and Glu, i.e., negatively charged AAs with short sidechains. Especially in these short 25-mer polypeptides, charged residues are likely to be on the surface. However, by having surface-anions and surface-cations in such close proximity, a polypeptide would be inclined to fold in such a way to favour formation of ion-pair salt bridges (Figure 6B, right). The backbone angles required to form such salt bridges would create additional constraints that might be hard to simultaneously satisfy along with hydrophobic packing, hence the ion-pairs would stabilize the protein in an unpacked (and therefore unfolded) conformation. Naturally, there are two ways to avoid this folding problem: Either (1) place positive charges further away from the backbone (on a longer sidechain) so that they will primarily interact by hydrogen bonding with water in a distinct solvation shell; *or* (2) do not include positively charged residues at all (Figure 6). We hypothesize that early proteins avoided ion pair-induced unfolding using the second strategy, whereas modern proteins adopted the first, though this strategy only became available once Arg and Lys could be biosynthesized. One experiment that could further test this hypothesis is the synthesis of a counterfactual peptide library in which basic residues use short side chains (diaminobutyric acid) whilst acidic residues use long side chains (e.g., 2-aminoadipic acid). Our model would predict that such a combination would also support secondary structure formation.



**Figure 6.** Biophysical models for the detrimental effect of unbranched hydrophobic sidechains (A) and short-chained basic residues (B) on folding stability of small proteins. (A) Unbranched hydrophobic residues render core packing more entropically expensive. (B) Short-chained basic residues occupy the same solvation sphere as canonical acidic residues, increasing the frequency of salt bridges over solvation with water.

In summary, our study on the solubility and secondary structure propensity of several prebiotically-relevant amino acid alphabets supports the assertion that foldability played a critical early role in governing which amino acids ultimately became part of the canonical alphabet. Unbranched amino acids and short-chain basic amino acids were excluded, despite their prebiotic abundance because they ‘over-solubilized’ polypeptides by stabilizing their unfolded conformations. More broadly, this study supports the view that the early canonical alphabet (10E), despite its deficiencies in relation to the modern canonical alphabet, was remarkably adaptive at supporting folding for the earliest proteins.

## EXPERIMENTAL PROCEDURES

All chemicals if not stated otherwise were purchased from Sigma Aldrich.

**Design and preparation of combinatorial peptide libraries.** Split and mix synthesis of peptide libraries has its limitations.<sup>42</sup> Complete library of 25-mers composed of 10 amino acids would in theory require  $10^{25}$  beads. One gram of solid phase support contains about  $10^6$  beads – therefore it would cover only  $10^{-21}\%$  of possible structures. To increase the probability of

completeness of the possible structures it is necessary to conduct the synthesis considering individual molecules.

Average molecular weight of a 25-mer peptide composed of 10 amino acids is about 2,500 Daltons. Therefore 2.5 kg of these peptides would potentially contain  $6 \times 10^{23}$  individual peptides. In order to have a chance to synthesize a complete library, we would need 250 kg of peptides, where each peptide would be present only once. But the chance of finding any given peptide in the mixture is about 70%. Therefore, studying a complete library is practically impossible.

We decided to synthesize the peptide mixture on 200 mg of the solid support and therefore create theoretically only  $10^{-4}\%$  of possible structures. The approach using individual molecules is still capable of production of  $10^{17}$ × more possible structures than split and mix strategy. However, considering complete randomness of the synthetic process, we concluded that the sample is a representative collection of possible structures. One mg of synthetic peptide mixture contains about  $2.4 \times 10^{18}$  individual peptides.

Synthesis of the peptide mixture was accomplished by coupling mixtures of amino acids in which the ratio of individual amino acids was adjusted according to their reactivities. This approach was used by several authors, and various amino acid ratios were reported.<sup>43</sup> We have used as the basis the ratios reported by Santi et al.<sup>44,45</sup> and adjusted the ratios for unnatural amino acids based on their structure and our previous experience (Supplementary Table S1).

Synthesis was performed on the automatic peptide synthesizer Spyder Mark IV, using standard Fmoc peptide synthesis protocol.<sup>46</sup> Fmoc amino acids were individually weighted (see Supplementary Table S1), mixed together and dissolved to create 0.3 M solution in 0.3 M N-hydroxybenzotriazole (HOBt) in dimethylformamide (DMF). Rink resin (200 mg, 0.42 mmol/g) was distributed into 10 ml syringes of the synthesizer and swelled in DMF for 10 minutes. The mixtures from Supplementary Table S1 were defined as extra amino acids 1 to 6 and placed into amino acid containers 21 to 26. Synthetic protocol was as follows: 2 × 1 min washing with DMF, 1 × 1 min and 1 × 20 min treatment with 20% piperidine in DMF, 4 × 1 min wash with DMF, 1 × 1 min wash with 0.3 M HOBt, 2 × 60 min coupling with amino acid mixture and 1 M diisopropylcarbodiimide in DMF. After 25 cycles of the synthesis, the resin was washed with DMF and dichloromethane and dried *in vacuo*. Dried resin was treated with 5 ml Mixture K (trifluoroacetic acid-thioanisole-water-phenol-ethanedithiol, 82.5:5:5:5:2.5, v/v) for 2 hours.<sup>47</sup> Resin was filtered off and peptide mixture was precipitated with diethyl ether three times and dried *in vacuo*. The pellet was then dissolved in 20% acetic acid and lyophilized. Prior to further experiments, all the peptide libraries were lyophilized three times with 1 mM HCl overnight.

**Quality control of peptide libraries.** The molecular weight distributions of combinatorial peptide libraries were confirmed by mass spectrometry using UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker, Germany) according to the standard procedure.

Prior to the amino acid analysis, the library samples were hydrolyzed in 6 M hydrochloric acid at 110 °C for 20 h, the hydrolysate was evaporated, and reconstituted with 0.1 M hydrochloric acid containing the internal standard. Amino acid analysis was performed on an Agilent 1260 HPLC (Agilent Technologies) equipped with a fluorescence detector using automated o-

phtalaldehyde/2-mercaptopropionic acid (OPA/MPA) or ninhydrin derivatization in case of 10E, 10U, 11R and 11D, 11Y, 19F libraries, respectively.

The presence of 2,4-diaminobutyric acid in the 11D library was confirmed by fluorescamine assay.<sup>48</sup> For the assay, peptide library solution (75  $\mu$ l) in PBS buffer was incubated with 25  $\mu$ l of fresh stock of 3 mg/ml fluorescamine in DMSO at room temperature for 1 hour in a 96-well plate (Greiner 650209). Fluorescence intensity ( $\lambda_{Ex}$  = 365 nm,  $\lambda_{Em}$  = 470 nm) was recorded using Tecan Spark plate reader. Primary amine concentrations were determined by linear interpolation of measured intensities for peptide H-GTIQPYPFSWGY-NH<sub>2</sub> in concentration range 87.5 – 2.7  $\mu$ M. Obtained amine concentrations were then divided by the concentration of the analyzed peptide determined by amino acid analysis to calculate the amount of amine equivalents per molecule. Experiments were conducted in duplicate.

**Solubility and aggregation propensity measurements.** A series of ten Britton-Robinson buffers at pH 3.0, 5.0, 7.4, 9.0, and 11.0 was prepared by mixing 20 mM acetic acid, 20 mM boric acid and 20 mM phosphoric acid and adjusting pH to the desired value with 5 M NaOH. The ionic strength was adjusted to either 50 mM (low ionic strength) or 500 mM (high ionic strength) with NaCl, and all Britton-Robinson buffers were filter-sterilized using 0.22  $\mu$ m PVDF membrane before use. Lyophilized peptide libraries were thoroughly resuspended in autoclaved MilliQ water to 5 mg/ml and subsequently diluted ten times into Britton-Robinson buffer to the final concentration of 0.5 mg/ml. The samples were gently shaken at room temperature for 30 min and then centrifuged at maximum speed (21,300  $\times$  g) for 15 min at 4  $^{\circ}$ C in order to remove insoluble fraction. The supernatant (soluble fraction) was taken and used for solubility and aggregation propensity analyses.

The relative amount of peptides in soluble fractions was estimated spectrophotometrically by absorption of peptide bonds at 215 nm for all peptide libraries except the 19F peptide library. Due to the extremely low solubility, solubility of 19F peptide library was estimated by fluorescence spectroscopy using Pierce Quantitative Fluorometric Peptide Assay (Thermo Fisher Scientific, USA), and 0.5 mg/ml solution in DMSO was used as a standard. Spectrophotometric measurements were performed five times, whereas fluorometric measurements were performed in triplicates.

The relative amount of soluble aggregates was estimated by size-exclusion chromatography. The 100  $\mu$ l aliquot of supernatant was loaded onto Superdex 75 Increase 10/300 GL column (Cytiva, USA) that was pre-equilibrated with two bed volumes of the corresponding 20 mM Britton-Robinson buffer. The peptides were eluted from the column by one bed volume of the buffer at 0.5 ml/min flow rate at room temperature, and the eluted peptides were detected by absorption at 215 nm.

**Circular Dichroism spectroscopy.** The CD spectra were recorded using a Chirascan-plus spectrophotometer (Applied Photophysics, UK) over the wavelength range 190–260 nm in steps of 1 nm with an averaging time of 1 sec per step. Cleared samples at 0.2 mg/ml nominal concentration in 1 mm path-length quartz cells were placed into a cell holder, and spectra were recorded at room temperature. The CD signal was obtained as ellipticity in units of millidegrees and the resulting spectra were averaged from two scans and buffer-spectrum subtracted. All CD measurements were performed twice, and the resulting ratio of ellipticity at



222 nm to ellipticity at 200 nm was averaged and used to estimate the secondary structure content in peptide libraries.

To estimate the effect of pH on secondary structure, 5 mg/ml suspensions of peptide libraries in MilliQ water were diluted into 10 mM Britton-Robinson buffers at pH 3.0; 5.0; 7.4; 9.0 and 11.0 to the final concentration of 0.2 mg/ml, gently mixed at room temperature for 30 min and then centrifuged at maximum speed ( $21,300 \times g$ ) for 15 min at 4 °C in order to remove insoluble fraction.

To estimate the effect of 2,2,2-trifluoroethanol on secondary structure, 5 mg/ml suspensions of peptide libraries in MilliQ water were diluted in a series of 10 mM Britton-Robinson buffer (pH 7.4) containing 0–90% (v/v) 2,2,2-trifluoroethanol.

To estimate the effect of metal ions on secondary structure, 5 mg/ml suspensions of peptide libraries in MilliQ water were diluted in a series of 10 mM Tris buffer at pH 7.4 supplemented with 0; 10; 100; and 1,000  $\mu$ M mixture of NaCl, KCl, MgCl<sub>2</sub>, MnCl<sub>2</sub>, and ZnCl<sub>2</sub>.

**Bioinformatic analysis of in silico peptide libraries.** 1,000 25-mer sequences were randomly generated *in silico* for the libraries and their structure was predicted using PEPstrMOD.<sup>31</sup> PEPstrMOD uses force field libraries Forcefield\_NCAA and Forcefield\_PTM and integrates them in Molecular Dynamics (MD) simulations using AMBER. Additionally, it implements the force field library SwissSideChain with the MD package GROMACS. The parameters employed in the MD simulations were 50 picoseconds of simulation time and the selected peptide environment was set to “vacuum”.

DSSP<sup>49</sup> was employed to determine the secondary structure elements from the peptide tertiary structures generated by PEPstrMOD. The obtained annotations of secondary structure were grouped into three larger classes: helix (H: alpha-helix, G: 310 helix and I:  $\pi$ -helix), sheet (B: isolated  $\beta$ -bridge and E: extended strand) and loop (T: hydrogen bonded turn, S: bend and “\_” blank spaces).

## ASSOCIATED CONTENT

**Supporting Information.** Supplementary Table S1 and Supplementary Figures S1-S6.

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## Notes

The authors declare no competing interests.

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## Supplementary Information

### Early selection of the amino acid alphabet was adaptively shaped by biophysical constraints of foldability

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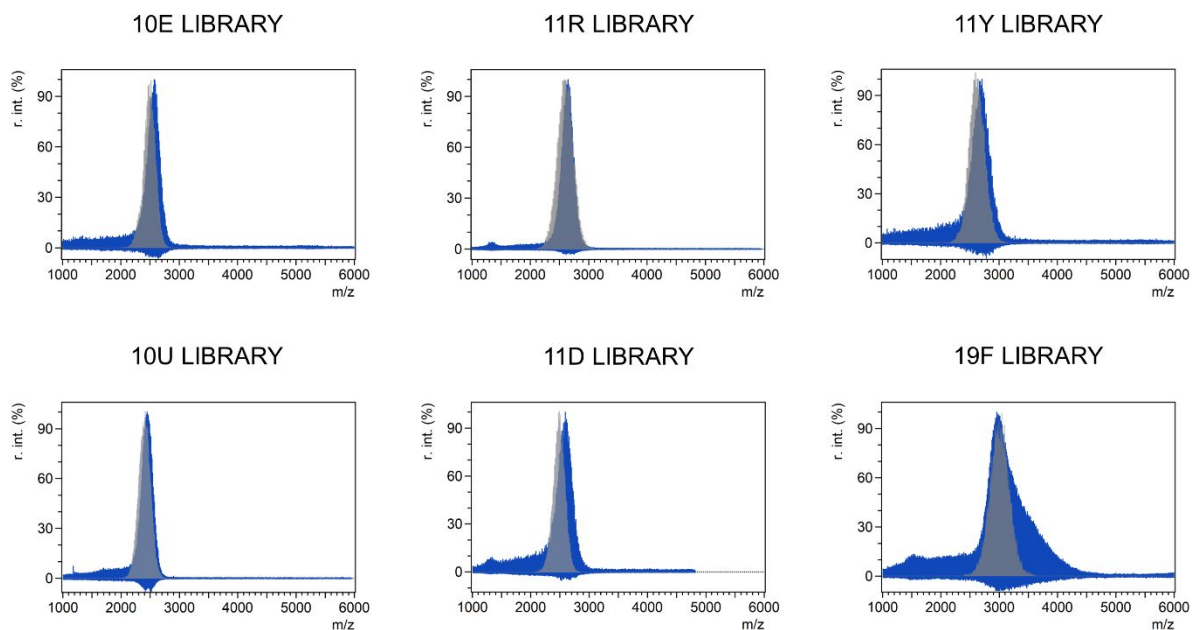
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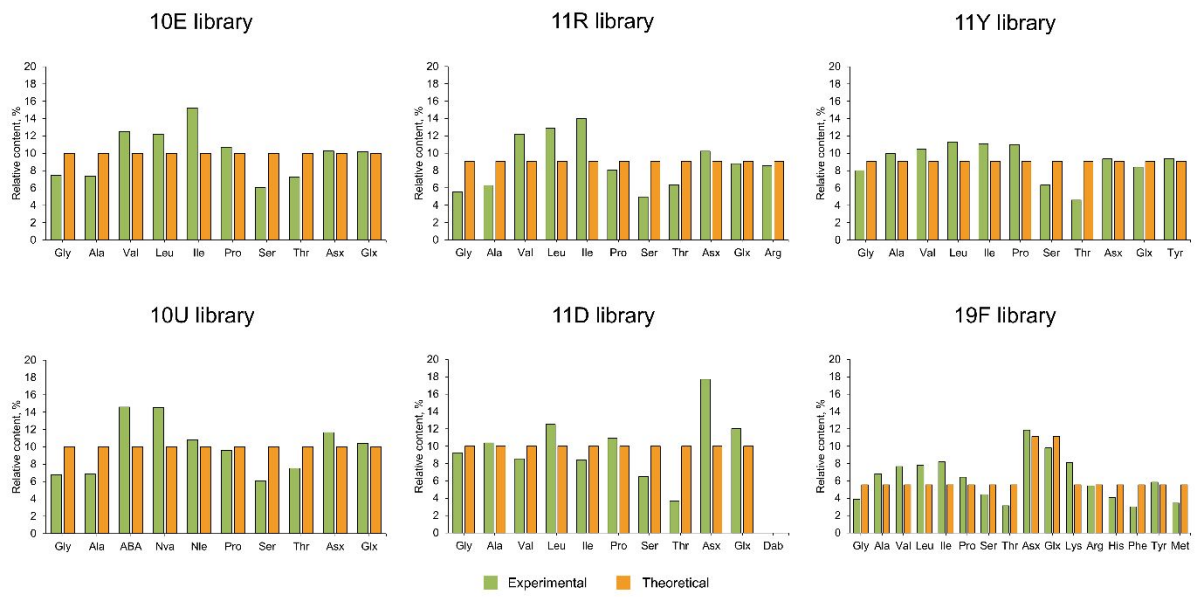
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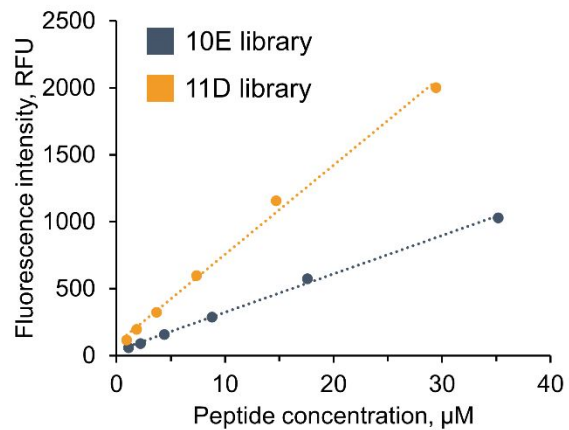
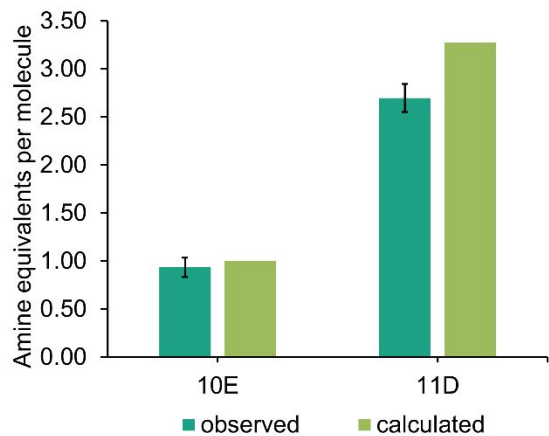




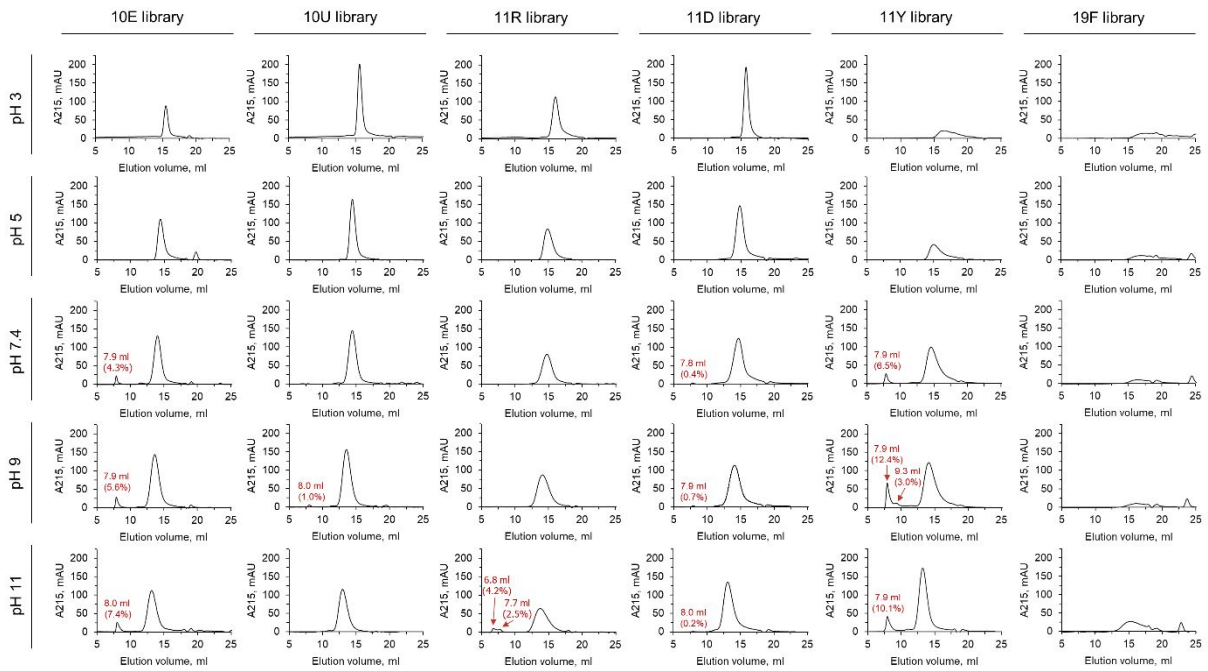
*Supplementary Figure S1. Molecular weight distribution of 25-mer combinatorial peptide libraries estimated by MALDI-TOF-MS using 2,5-dihydrobenzoic acid (DHB) matrix. Observed m/z distribution is shown in blue, expected m/z distribution is shown as a gray overlay. Peptide libraries were dissolved in acetonitrile:water (1:1) mixture + 0.1 % (v/v) acetic acid. 19F peptide library was dissolved in acetonitrile:water (1:1) mixture.*



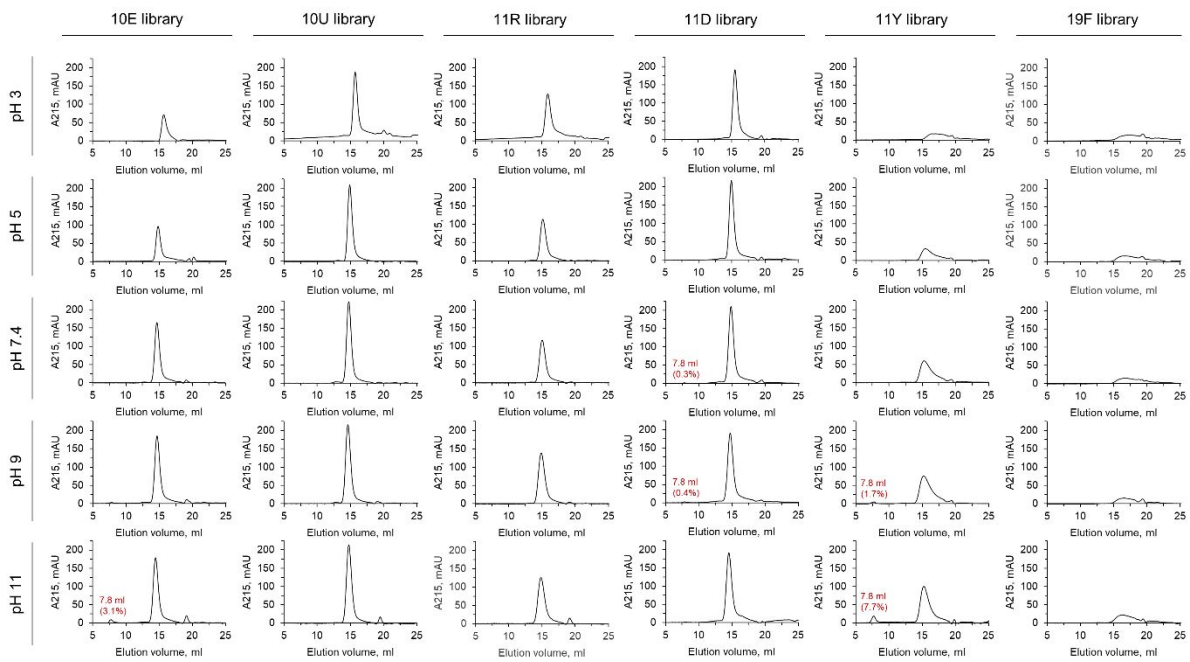
*Supplementary Figure S2. Amino acid composition of 25-mer combinatorial peptide libraries determined by amino acid analysis.*



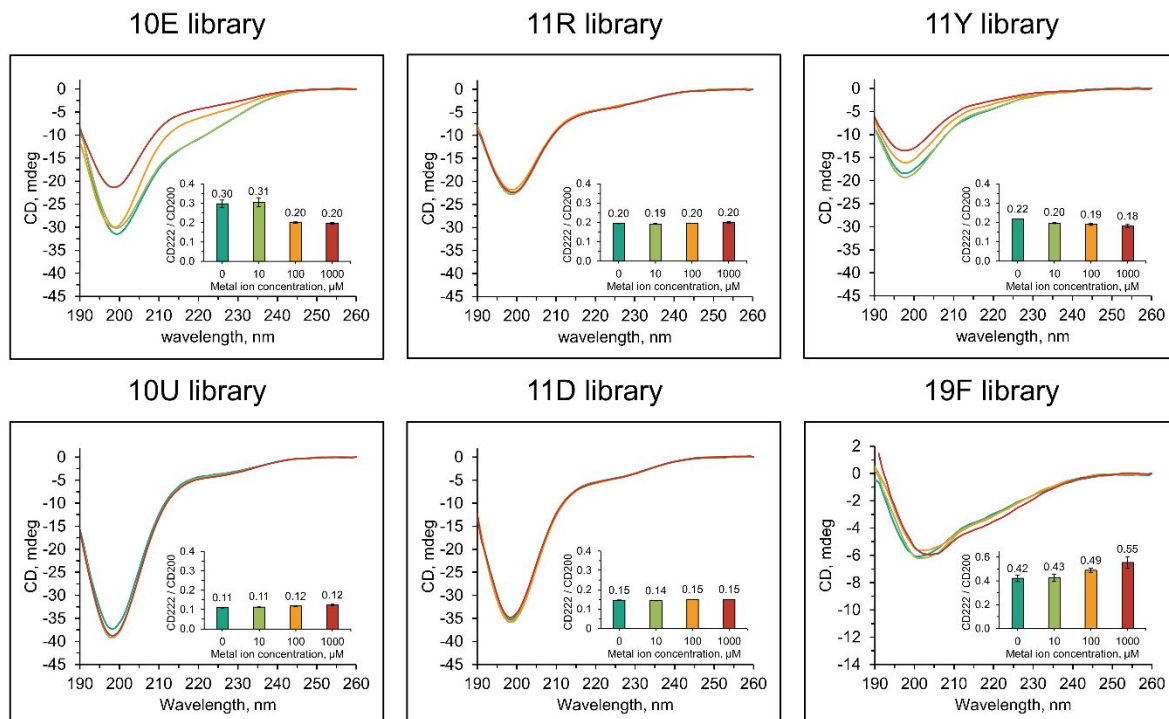
*Supplementary Figure S3. The quantification of free amino acid groups in 10E and 11D peptide libraries using fluorescamine assay.*



**Supplementary Figure S4. Aggregation propensity of 25-mer combinatorial peptide libraries at different pH and low ionic strength (50 mM NaCl). Aggregation propensity was measured for 0.5 mg/ml nominal peptide library solutions in a series of 20 mM ABP buffers (pH 3–11) by size-exclusion chromatography.**



**Supplementary Figure S5. Aggregation propensity of 25-mer combinatorial peptide libraries at different pH and high ionic strength (500 mM NaCl). Aggregation propensity was measured for 0.5 mg/ml nominal peptide library solutions in a series of 20 mM ABP buffers (pH 3–11) by size-exclusion chromatography.**



**Supplementary Figure S6.** The effect of metal ions on the secondary structure of 25-mer combinatorial peptide libraries. CD spectra were measured for 0.2 mg/ml nominal peptide library solutions in a series of 10 mM Tris buffers at pH 7.4 supplemented with 0 (green line); 10 (yellow line); 100 (orange line); and 1,000 (red line)  $\mu\text{M}$  mixture of NaCl, KCl,  $\text{MgCl}_2$ ,  $\text{MnCl}_2$ , and  $\text{ZnCl}_2$ . The inset graph shows the ratios of CD signal at 222 to 200 nm.

*Supplementary Table S1: Weights (mg) and amounts (mmol) of protected amino acids in individual isokinetic mixtures used for synthesis. Mixtures were dissolved in a final volume listed in the last row yielding 0.3 M solution of amino acids in 0.3 M HOBt in DMF.*

	19F		10E		11R		11Y		10U		11D	
	m	n	m	n	m	n	m	n	m	n	m	n
	mg	mmol	mg	mmol	mg	mmol	mg	mmol	mg	mmol	mg	mmol
Fmoc-Ala	202	0.61	332	1.01	300	0.91	312	0.95	508	1.54	303	0.92
Fmoc-Asp(OtBu)	276	0.67	455	1.11	409	0.99	426	1.04	694	1.69	414	1.01
Fmoc-Glu(OtBu)	296	0.67	489	1.10	440	0.99	457	1.03	745	1.68	444	1.00
Fmoc-Phe	187	0.48										
Fmoc-Gly	164	0.55	270	0.91	243	0.82	253	0.85	412	1.39	246	0.83
Fmoc-His(Trt)	422	0.68										
Fmoc-Ile	1,171	3.31	1,934	5.47	1,740	4.92	1,809	5.12			1,759	4.98
Fmoc-Lys(Boc)	556	1.19										
Fmoc-Leu	335	0.95	553	1.56	497	1.41	517	1.46			503	1.42
Fmoc-Met	163	0.44										
Fmoc-Asn(Trt)	609	1.02										
Fmoc-Pro	278	0.78	460	1.29	414	1.16	430	1.21	702	1.98	418	1.18
Fmoc-Gln(Trt)	620	1.02										
Fmoc-Arg(Pmc)	807	1.22			1,199	1.81						
Fmoc-Ser(tBu)	203	0.53	336	0.88	302	0.79	314	0.82	512	1.34	306	0.80
Fmoc-Thr(tBu)	363	0.91	599	1.51	539	1.36	560	1.41	914	2.30	545	1.37
Fmoc-Val	731	2.15	1,208	3.56	1,086	3.20	1,130	3.33			1,099	3.24
Fmoc-Trp(Boc)	254	0.48										
Fmoc-Tyr(tBu)	363	0.79					560	1.22				
Fmoc-Nva									752	2.22		
Fmoc-Nle									775	2.19		
Fmoc-Aba									648	1.99		
Fmoc-Dab(Boc)											734	1.67
V / ml	61.5		61.3		61.2		61.4		39.7		55.8	

Supplementary table S2. Statistical analysis of relative solubilities at different pH and ionic strengths. The tables include ratios of relative solubilities and P-values. P-values are calculated using a two-tailed Student's t-test and assuming homoscedasticity.

(a) Statistical analysis of solubilities at low (50 mM NaCl) vs high (500 mM NaCl) ionic strengths

		10E	10U	11R	11D	11Y	19F
pH 3.0	Ratio	1.0264	1.0285	1.1484	1.0682	1.0560	1.4677
	P-value	0.3448	0.3946	0.0564	0.0214	0.4727	0.0172
pH 5.0	Ratio	0.9580	1.0137	0.9736	0.9656	0.9895	1.4433
	P-value	0.6268	0.6617	0.7776	0.0395	0.8594	0.0226
pH 7.4	Ratio	1.2833	0.9966	1.0013	0.9875	1.6230	1.0857
	P-value	0.0002	0.8585	0.9781	0.4112	0.0031	0.2538
pH 9.0	Ratio	0.9979	1.0239	1.1019	0.9945	1.4544	1.0753
	P-value	0.8370	0.1289	0.0263	0.4117	0.0160	0.5570
pH 11.0	Ratio	0.9881	0.9957	1.3588	1.0231	1.1160	0.9891
	P-value	0.4577	0.6999	2.1E-5	0.0546	0.4751	0.8085

(b) Statistical analysis of solubilities at low (50 mM NaCl) ionic strength at pH 3.0

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	0.4673					
	P-value	2.1E-5					
11R	Ratio	0.5790	1.2391				
	P-value	4.0E-5	0.0028				
11D	Ratio	0.3778	0.8085	0.6524			
	P-value	3.3E-7	0.0007	3.1E-5			
11Y	Ratio	2.2921	4.9050	3.9584	6.0671		
	P-value	4.5E-6	4.7E-6	5.0E-6	1.5E-7		
19F	Ratio	0.9739	2.0842	1.6820	2.5780	0.4249	
	P-value	0.6288	0.0002	0.0007	2.0E-5	0.0008	

(c) Statistical analysis of solubilities at low (50 mM NaCl) ionic strength at pH 5.0

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	0.4997					
	P-value	0.0001					
11R	Ratio	0.8370	1.6750				
	P-value	0.0958	0.0008				
11D	Ratio	0.4947	0.9901	0.5911			
	P-value	4.9E-5	0.5904	0.0004			
11Y	Ratio	2.0150	4.0324	2.4075	4.0729		
	P-value	0.0009	4.9E-6	0.0008	2.5E-7		
19F	Ratio	1.3055	2.6127	1.5598	2.6389	0.6479	
	P-value	0.0309	3.4E-5	0.0081	1.0E-5	0.0044	



(d) Statistical analysis of solubilities at low (50 mM NaCl) ionic strength at pH 7.4

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	1.0024					
	P-value	0.9157					
11R	Ratio	1.4744	1.4709				
	P-value	0.0001	0.0001				
11D	Ratio	1.0098	1.0074	0.6849			
	P-value	0.6847	0.7210	0.0001			
11Y	Ratio	1.1801	1.1773	0.8004	1.1687		
	P-value	0.0025	0.0021	0.0052	0.0022		
19F	Ratio	4.1326	4.1229	2.8029	4.0927	3.5020	
	P-value	1.8E-6	1.1E-6	3.8E-5	1.0E-6	1.6E-5	

(e) Statistical analysis of solubilities at low (50 mM NaCl) ionic strength at pH 9.0

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	0.9998					
	P-value	0.9490					
11R	Ratio	1.2767	1.2769				
	P-value	4.6E-5	2.3E-5				
11D	Ratio	1.0094	1.0096	0.7907			
	P-value	0.5400	0.3905	6.7E-6			
11Y	Ratio	0.9979	0.9982	0.7817	0.9886		
	P-value	0.6588	0.6219	0.0006	0.8537		
19F	Ratio	3.9955	3.9963	3.1296	3.9582	4.0037	
	P-value	9.5E-6	8.5E-6	3.1E-5	7.8E-6	2.0E-5	

(f) Statistical analysis of solubilities at low (50 mM NaCl) ionic strength at pH 11.0

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	0.9840					
	P-value	0.3263					
11R	Ratio	0.9798	0.9957				
	P-value	0.0895	0.2452				
11D	Ratio	0.9798	0.9957	1.0000			
	P-value	0.1452	0.4924	0.2810			
11Y	Ratio	1.0313	1.0480	1.0526	1.0526		
	P-value	0.0860	0.0213	0.0070	0.0099		
19F	Ratio	3.4413	3.4971	3.5124	3.5124	3.3370	
	P-value	2.0E-6	8.9E-7	3.7E-7	4.5E-7	3.2E-6	

(g) Statistical analysis of solubilities at high (500 mM NaCl) ionic strength at pH 3.0

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	0.4683					
	P-value	1.6E-5					
11R	Ratio	0.6478	1.3835				
	P-value	0.0024	0.0027				
11D	Ratio	0.3932	0.8397	0.6069			
	P-value	4.9E-6	0.0019	0.0003			
11Y	Ratio	2.3581	5.0360	3.6400	5.9976		
	P-value	7.3E-5	3.5E-6	0.0002	1.5E-6		
19F	Ratio	1.3926	2.9741	2.1497	3.5420	0.5906	
	P-value	0.0096	3.1E-5	0.0010	1.1E-5	0.0073	

(h) Statistical analysis of solubilities at high (500 mM NaCl) ionic strength at pH 5.0

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	0.5287					
	P-value	0.0002					
11R	Ratio	0.8506	1.6087				
	P-value	0.1249	0.0010				
11D	Ratio	0.4986	0.9431	0.5862			
	P-value	9.6E-5	0.0519	0.0004			
11Y	Ratio	2.0812	3.9362	2.4468	4.1737		
	P-value	0.0012	4.8E-6	0.0007	6.7E-7		
19F	Ratio	1.9669	3.7201	2.3124	3.9446	0.9451	
	P-value	0.0031	2.1E-5	0.0015	7.9E-6	0.4946	

(i) Statistical analysis of solubilities at high (500 mM NaCl) ionic strength at pH 7.4

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	0.7784					
	P-value	0.0003					
11R	Ratio	1.1505	1.4779				
	P-value	0.0209	0.0004				
11D	Ratio	0.7770	0.9982	0.6754			
	P-value	9.2E-5	0.8350	0.0003			
11Y	Ratio	1.4926	1.9174	1.2974	1.9208		
	P-value	0.0050	0.0006	0.0330	0.0005		
19F	Ratio	3.4963	4.4915	3.0391	4.4995	2.3425	
	P-value	1.6E-6	1.4E-6	5.7E-5	4.5E-7	0.0034	

(j) Statistical analysis of solubilities at high (500 mM NaCl) ionic strength at pH 9.0

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	1.0257					
	P-value	0.0698					
11R	Ratio	1.4097	1.3743				
	P-value	0.0001	0.0003				
11D	Ratio	1.0059	0.9807	0.7136			
	P-value	0.6154	0.1297	0.0002			
11Y	Ratio	1.4544	1.4179	1.0318	1.4459		
	P-value	0.0125	0.0168	0.6691	0.0130		
19F	Ratio	4.3053	4.1973	3.0542	4.2800	2.9602	
	P-value	3.5E-7	1.1E-6	3.4E-5	5.1E-7	0.0041	

(k) Statistical analysis of solubilities at high (500 mM NaCl) ionic strength at pH 11.0

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	0.9916					
	P-value	0.4210					
11R	Ratio	1.3474	1.3588				
	P-value	5.4E-5	4.1E-5				
11D	Ratio	1.0145	1.0231	0.7529			
	P-value	0.3492	0.1158	6.6E-5			
11Y	Ratio	1.1647	1.1746	0.8644	1.1481		
	P-value	0.2884	0.2646	0.4659	0.3213		
19F	Ratio	3.4451	3.4743	2.5568	3.3959	2.9578	
	P-value	1.4E-7	9.6E-8	3.3E-6	1.6E-7	0.0111	

Supplementary table S3. Statistical analysis of CD222/CD200 values at different pH values. The tables include ratios of CD222/CD200 values and P-values. P-values are calculated using a two-tailed Student's t-test and assuming homoscedasticity.

(a) Statistical analysis of CD222/CD200 values at pH 3.0

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	1.4454					
	P-value	0.0102					
11R	Ratio	1.0564	0.7309				
	P-value	0.2795	0.0054				
11D	Ratio	1.3374	0.9253	1.2660			
	P-value	0.0159	0.0213	0.0102			
11Y	Ratio	0.9235	0.6389	0.8741	0.6905		
	P-value	0.2158	0.0076	0.0730	0.7528		
19F	Ratio	0.7829	0.5416	0.7410	0.5853	0.8477	
	P-value	0.0796	0.0170	0.0531	0.9173	0.1479	

(b) Statistical analysis of CD222/CD200 values at pH 5.0

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	1.5078					
	P-value	0.0059					
11R	Ratio	1.0490	0.6957				
	P-value	0.1904	0.0012				
11D	Ratio	1.3382	0.8875	1.2757			
	P-value	0.0101	0.0249	0.0019			
11Y	Ratio	1.0940	0.7255	1.0429	0.8175		
	P-value	0.0928	0.0051	0.1186	0.5937		
19F	Ratio	0.5058	0.3354	0.4822	0.3379	0.4623	
	P-value	0.0760	0.0440	0.0695	0.6946	0.0651	

(c) Statistical analysis of CD222/CD200 values at pH 7.4

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	3.0516					
	P-value	0.0004					
11R	Ratio	1.7835	0.5844				
	P-value	0.0017	0.0056				
11D	Ratio	2.4441	0.8009	1.3704			
	P-value	0.0003	0.0118	0.0101			
11Y	Ratio	1.2538	0.4109	0.7030	0.5130		
	P-value	0.0037	0.0006	0.0052	0.9884		
19F	Ratio	1.5215	0.4986	0.8531	0.6225	1.2135	
	P-value	0.0122	0.0130	0.1373	0.8600	0.0655	

(d) Statistical analysis of CD222/CD200 values at pH 9.0

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	2.3852					
	P-value	0.0006					
11R	Ratio	0.9834	0.4123				
	P-value	0.2634	0.0002				
11D	Ratio	1.9297	0.8091	1.9623			
	P-value	0.0005	0.0096	3.0E-5			
11Y	Ratio	0.9257	0.3881	0.9413	0.4797		
	P-value	0.3894	0.0121	0.4754	0.9183		
19F	Ratio	0.9682	0.4059	0.9845	0.5017	1.0459	
	P-value	0.2465	0.0010	0.4470	0.9906	0.5910	

(e) Statistical analysis of CD222/CD200 values at pH 11.0

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	2.2522					
	P-value	0.0003					
11R	Ratio	0.7160	0.3179				
	P-value	0.0240	0.0043				
11D	Ratio	1.9858	0.8817	2.7734			
	P-value	0.0002	0.0204	0.0049			
11Y	Ratio	0.9523	0.4228	1.3301	0.4796		
	P-value	0.0564	0.0005	0.0317	0.9635		
19F	Ratio	0.9157	0.4066	1.2790	0.4611	0.9616	
	P-value	0.0088	0.0002	0.0397	0.9408	0.0774	

Supplementary table S4. Statistical analysis of CD222/CD200 values at different 2,2,2-trifluoroethanol concentrations. The tables include ratios of CD222/CD200 values and P-values. P-values are calculated using a two-tailed Student's t-test and assuming homoscedasticity.

(a) Statistical analysis of CD222/CD200 values at 0% (v/v) 2,2,2-trifluoroethanol

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	3.0516					
	P-value	0.0004					
11R	Ratio	1.7910	0.5869				
	P-value	0.0013	0.0045				
11D	Ratio	2.4441	0.8009	1.3647			
	P-value	0.0003	0.0118	0.0075			
11Y	Ratio	1.2538	0.4109	0.7000	0.5130		
	P-value	0.0037	0.0006	0.0039	0.9884		
19F	Ratio	1.6823	0.5513	0.9393	0.6883	1.3417	
	P-value	0.0560	0.1169	0.7543	0.8254	0.1796	

(b) Statistical analysis of CD222/CD200 values at 10% (v/v) 2,2,2-trifluoroethanol

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	3.3216					
	P-value	0.0005					
11R	Ratio	2.0610	0.6205				
	P-value	0.0030	0.0154				
11D	Ratio	2.8413	0.8554	1.3786			
	P-value	0.0006	0.0032	0.0292			
11Y	Ratio	1.2974	0.3906	0.6295	0.4566		
	P-value	0.0090	0.0010	0.0092	0.9237		
19F	Ratio	1.6901	0.5088	0.8201	0.5948	1.3027	
	P-value	0.0027	0.0023	0.0588	0.8806	0.0128	

(c) Statistical analysis of CD222/CD200 values at 20% (v/v) 2,2,2-trifluoroethanol

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	2.6675					
	P-value	0.0001					
11R	Ratio	1.6638	0.6237				
	P-value	0.0006	0.0025				
11D	Ratio	2.3442	0.8788	1.4089			
	P-value	0.0002	0.0341	0.0047			
11Y	Ratio	0.8460	0.3171	0.5085	0.3609		
	P-value	0.0032	0.0002	0.0006	0.7995		
19F	Ratio	0.6663	0.2498	0.4005	0.2843	0.7876	
	P-value	0.0005	0.0001	0.0003	0.7014	0.0023	

(d) Statistical analysis of CD222/CD200 values at 30% (v/v) 2,2,2-trifluoroethanol

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	2.2870					
	P-value	4.9E-5					
11R	Ratio	1.2366	0.5407				
	P-value	0.0074	0.0021				
11D	Ratio	1.9516	0.8534	1.5782			
	P-value	0.0003	0.0138	0.0038			
11Y	Ratio	0.6514	0.2849	0.5268	0.3338		
	P-value	0.0007	0.0002	0.0009	0.7630		
19F	Ratio	0.2595	0.1134	0.2098	0.1330	0.3983	
	P-value	0.0097	0.0068	0.0085	0.4754	0.0146	

(e) Statistical analysis of CD222/CD200 values at 40% (v/v) 2,2,2-trifluoroethanol

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	2.1465					
	P-value	0.0015					
11R	Ratio	1.0300	0.4799				
	P-value	0.5512	0.0055				
11D	Ratio	1.8074	0.8420	1.7547			
	P-value	0.0019	0.0123	0.0076			
11Y	Ratio	0.5943	0.2769	0.5770	0.3288		
	P-value	0.0032	0.0008	0.0048	0.7472		
19F	Ratio	0.1359	0.0633	0.1320	0.0752	0.2287	
	P-value	0.0184	0.0157	0.0182	0.3865	0.0229	

(f) Statistical analysis of CD222/CD200 values at 50% (v/v) 2,2,2-trifluoroethanol

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	2.0240					
	P-value	0.0010					
11R	Ratio	0.9415	0.4652				
	P-value	0.2231	0.0036				
11D	Ratio	1.6898	0.8349	1.7948			
	P-value	0.0014	0.0136	0.0052			
11Y	Ratio	0.5706	0.2819	0.6060	0.3377		
	P-value	0.0007	0.0002	0.0027	0.7691		
19F	Ratio	0.1174	0.0580	0.1246	0.0694	0.2057	
	P-value	0.0008	0.0007	0.0008	0.4596	0.0010	

*(g) Statistical analysis of CD222/CD200 values at 60% (v/v) 2,2,2-trifluoroethanol*

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	1.9533					
	P-value	0.0010					
11R	Ratio	0.9008	0.4612				
	P-value	0.0515	0.0016				
11D	Ratio	1.6126	0.8256	1.7901			
	P-value	0.0019	0.0146	0.0026			
11Y	Ratio	0.5535	0.2834	0.6145	0.3433		
	P-value	0.0003	4.2E-5	0.0011	0.7827		
19F	Ratio	0.1113	0.0570	0.1236	0.0690	0.2011	
	P-value	8.0E-6	5.7E-6	1.4E-5	0.4779	6.7E-6	

*(h) Statistical analysis of CD222/CD200 values at 70% (v/v) 2,2,2-trifluoroethanol*

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	1.8746					
	P-value	0.0025					
11R	Ratio	0.8534	0.4553				
	P-value	0.0475	0.0029				
11D	Ratio	1.5377	0.8203	1.8019			
	P-value	0.0037	0.0106	0.0041			
11Y	Ratio	0.5533	0.2952	0.6483	0.3598		
	P-value	0.0007	0.0001	0.0028	0.8018		
19F	Ratio	0.0961	0.0513	0.1126	0.0625	0.1736	
	P-value	0.0049	0.0044	0.0051	0.4219	0.0058	

*(i) Statistical analysis of CD222/CD200 values at 80% (v/v) 2,2,2-trifluoroethanol*

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	1.7646					
	P-value	0.0070					
11R	Ratio	0.8485	0.4809				
	P-value	0.0632	0.0039				
11D	Ratio	1.4690	0.8325	1.7312			
	P-value	0.0104	0.0245	0.0049			
11Y	Ratio	0.5381	0.3049	0.6341	0.3663		
	P-value	0.0014	0.0002	0.0026	0.8132		
19F	Ratio	0.0734	0.0416	0.0864	0.0499	0.1363	
	P-value	0.0011	0.0010	0.0011	0.4384	0.0012	



*(j) Statistical analysis of CD222/CD200 values at 90% (v/v) 2,2,2-trifluoroethanol*

		10E	10U	11R	11D	11Y	19F
10E	Ratio						
	P-value						
10U	Ratio	1.6794					
	P-value	0.0091					
11R	Ratio	0.8393	0.4997				
	P-value	0.0550	0.0029				
11D	Ratio	1.3614	0.8106	1.6221			
	P-value	0.0213	0.0201	0.0052			
11Y	Ratio	0.5214	0.3105	0.6213	0.3830		
	P-value	0.0143	0.0063	0.0217	0.7891		
19F	Ratio	0.0184	0.0110	0.0220	0.0135	0.0353	
	P-value	0.0270	0.0266	0.0272	0.3064	0.0279	