



# Machine learning for evolutionary-based and physics-inspired protein design: Current and future synergies

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## Abstract

Computational protein design facilitates the discovery of novel proteins with prescribed structure and functionality. Exciting designs were recently reported using novel data-driven methodologies that can be roughly divided into two categories: evolutionary-based and physics-inspired approaches. The former infer characteristic sequence features shared by sets of evolutionary-related proteins, such as conserved or coevolving positions, and recombine them to generate candidates with similar structure and function. The latter approaches estimate key biochemical properties, such as structure free energy, conformational entropy, or binding affinities using machine learning surrogates, and optimize them to yield improved designs. Here, we review recent progress along both tracks, discuss their strengths and weaknesses, and highlight opportunities for synergistic approaches.

## Addresses

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## Introduction

Recent years have witnessed tremendous developments in computational protein design methodologies, following two parallel tracks: evolutionary-based design and physics-inspired design. The former has been propelled by (i) increasingly fast sequencing and homology

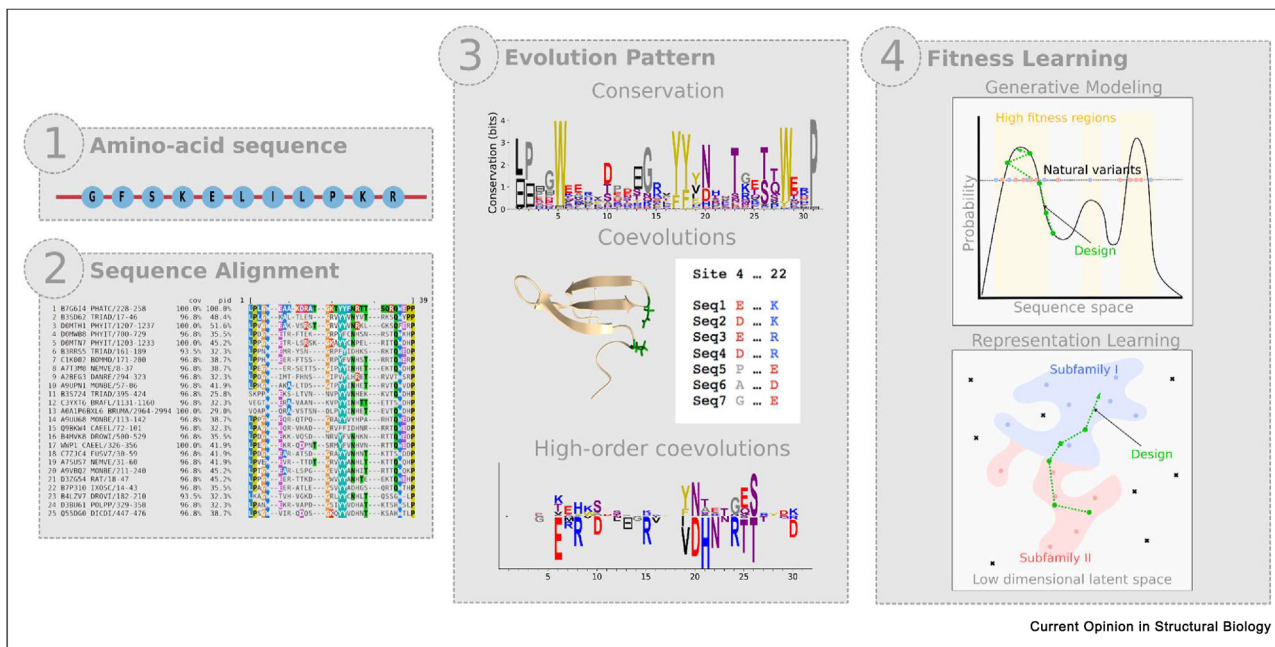
detection methodologies, allowing the constitution of large sequence databases, such as Uniprot [1], structured into families of evolutionary-related proteins, such as PFAM [2], and (ii) novel unsupervised machine learning approaches for generative modeling of sequences (see review by Wu et al. [3]). The latter have been stirred by the emergence of a variety of deep learning models for predicting properties of proteins from sequence, structure, or both (see review by Ovchinnikov and Huang [4]). Such prediction-based design protocols are highly appealing compared to traditional physics-based protein design protocols based on *e.g.* Rosetta or FoldX. Indeed, they circumvent two fundamental challenges of physics-based methods: (i) the necessity to extensively sample the structure conformation space to estimate thermodynamic quantities and (ii) the high computational cost of exploring the vast sequence space. Although evolutionary-based and physics-inspired machine learning methodologies are not systematically combined, they are highly synergistic in multiple aspects. Accordingly, the complementarity between evolutionary and physical modalities was successfully demonstrated for non-machine learning-based methods (see reviews by Marques et al., 2021 and Weinstein et al., 2020 [5,6]) (Figure 2).

Here, we will review recent achievements for both evolutionary-based and physics-inspired methods with an emphasis on experimentally validated works. We will discuss the current limitations of both approaches and complementarities between them. Finally, we will review recent works combining both approaches and highlight possible future directions.

## Evolutionary-based design

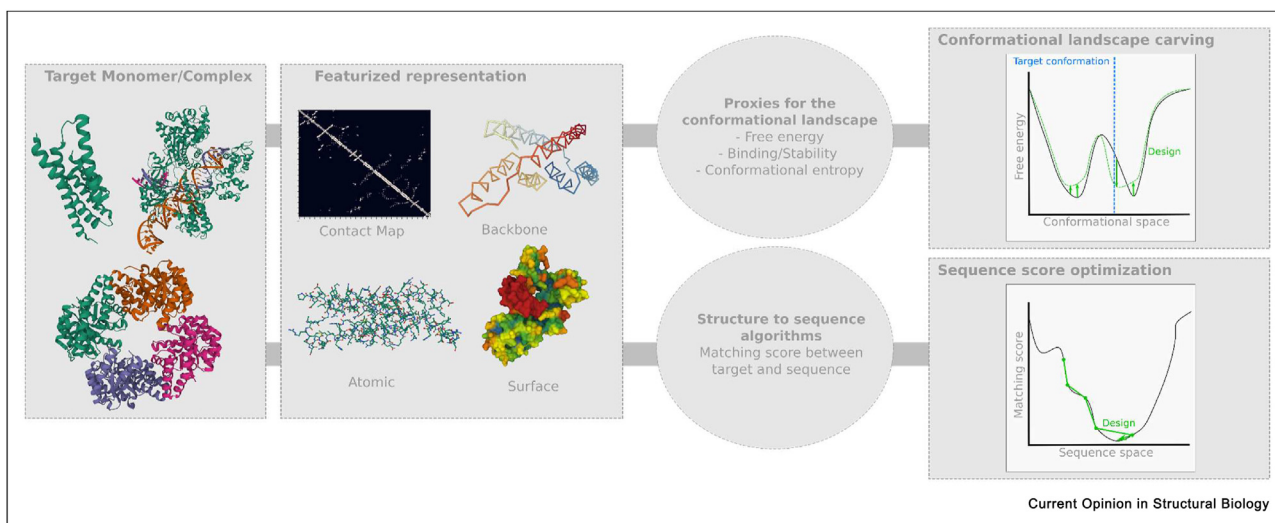
Numerous design protocols involve the modification of a preexisting natural protein toward improved or novel functional properties. Significant optimization of the target property(ies) requires exploring sequences harboring many mutations from the wild-type protein. However, it is estimated that up to 50% of single-point mutations are deleterious [7] for function, leading to exponentially decreasing success rates when mutating multiple sites. One solution is to restrict the search to mutations or combinations of mutations previously encountered, or likely to be encountered, throughout

Figure 1



**Main steps of evolutionary-based design.** Starting from an amino-acid sequence, homologous sequences are retrieved from sequence databases and a multiple sequence alignment (MSA) is constructed. MSAs feature various evolutionary patterns, including conservation, pairwise coevolution, and high-order coevolution, that reflect structural and functional constraints. Unsupervised machine learning distills fitness landscapes and representations from the evolutionary patterns, which, in turn, can be used for design.

Figure 2



**Main steps of physics-inspired design.** Starting from a (partially or fully prescribed) target structure, one first builds a featurized representation suitable for relevant deep learning algorithms. Next, two complementary approaches are possible. First, sequence-to-structure prediction algorithms can be leveraged to build proxies for the free energy landscape. The latter is then used to design a sequence whose free energy minimum lies at the target conformation. Second, structure-to-sequence algorithms can be used to generate suitable sequences from structure. They rely on a matching score between target and sequence that can be optimized to find suitable sequences.

the natural evolution of the protein. The evolutionary-based design consists of three main steps: (i) the collection and alignment of a set of sequences homologous to the wild-type protein that share similar structure and function, (ii) the construction of a statistical/machine learning model that captures common patterns shared between these sequences, such as conservation and coevolution, and (iii) the generation of artificial sequences that are distinct from natural ones but preserve the common patterns.

While early models, such as position-specific scoring matrices (PSSM), solely focused on capturing site-specific amino acid frequencies, increasingly complex statistical models based on self-supervised machine learning have been developed. The direct coupling analysis (DCA) method captures both single-site and pairwise correlations [8–10] arising from coevolution, allowing modeling of epistatic effects and drastic reduction of the search space. For instance, Russ et al. [11] designed with DCA hundreds of diverse chorismate mutase enzymes with native-like functionality, with a high success rate (~30%). Based on the entropy of the sequence distributions, they estimated that  $10^{85}$  of all  $10^{125}$  possible sequences with the same length were potential design candidates based on a single-site model, whereas only  $10^{25}$  were suitable when including pairwise correlations.

Recent works investigated various neural network-based machine learning generative models (Box 1) [12–18], achieving successes in various enzyme and nanobody design tasks. These networks have more flexible probability distributions than PSSM or DCA models, allowing integration into the model of higher-order MSA

statistics, such as the co-occurrence of amino acid n-uplets. Importantly, some of these methods also learn a continuous, low-dimensional latent space representation of the protein. Unlike in the original sequence space—where single-point mutations may reduce the probability and abolish function—small motions in latent space generally preserve the probability and function. Thus, it is easier to navigate and learn sequence/function associations in such “hole-free” landscapes. In Ref. [19], Biswas et al. leveraged the latent representation of a language model (see below) and small-scale experimental data to design improved GFP variants. Lian et al. [20] used a variational autoencoder (a representation-based generative model) to generate synthetic sequences of yeast SH<sub>3</sub> domains and evaluated their ability to replace a native SH<sub>3</sub> domain *in vivo*. They found that most of the sequences with native-like functionality mapped to a local, convex region of the latent space.

Beyond the direct generation of novel protein sequences, evolutionary models can also predict fitness-improving mutations [12,21], guide library design for large-scale screening experiments [22,23], or, conversely, infer fitness landscape from directed evolution experiments [24].

One drawback of such family-level models is that they do not generalize across protein families and as such can only be applied to protein families that include a large number of sequences. This is particularly problematic for proteins only conserved in eukaryotes. One possible avenue for overcoming these limitations is protein language models, as they can simultaneously model unrelated sets of protein sequences. Trained on large, unannotated databases of protein sequences, such as UniClust [25] or BFD [26],

### Box 1. Generative models, autoregressive models, and representations

**Generative models** are parametric probability distributions over a high-dimensional space of the form  $P_{\theta}(s)$ , such that  $P_{\theta}(s) > 0$  for all  $S$  and  $\sum P_{\theta}(s) = 1$ . The set of parameters  $\theta$  are learned from the data by maximizing the average log probability  $\langle \log P_{\theta}(s) \rangle$ . Informally, this amounts to assigning high probability to observed sequences and low elsewhere (Figure 1:4, upper panel). After learning, new sequences are generated by drawing samples from the probability distribution.

**Autoregressive generate models** are a special class of generative models of the form  $P_{\theta}(s) = P_{\theta}(s_1)P_{\theta}(s_2|s_1)P_{\theta}(s_3|s_1, s_2)\dots$ . The conditional distributions  $P_{\theta}(s_j|s_{<j})$  are parameterized by neural networks. Autoregressive models can be trained by maximizing the maximum likelihood as above, or, more efficiently, by masked modeling. The latter consists of masking (i.e. replacing by a placeholder) a random subset of positions (10–20%) and predicting their value from the remaining unmasked positions, via the conditional distributions. Informally, masked modeling enables learning of the interdependencies between the variables. After training, samples are generated by recursively sampling the next amino acid of a sequence given all the previous ones.

A **representation** is any deterministic mapping  $R(S)$  from one high-dimensional space (e.g. the sequence space) to a continuous space of typically lower dimension. Representations are especially useful for modeling proteins sequences when key properties  $f(S)$  of the sequences (e.g. probability density, biological fitness, substrate specificity, and phylogeny) can be well approximated as  $f_R(R(S))$ , where  $f_R$  is a *continuous, smooth* function (Figure 1:4, lower panel). This contrasts with the original sequence space, where small changes in the sequence can result in large changes of the properties (e.g. a single mutation can abolish function). Representation can be learned either via unsupervised or supervised learning, by parameterizing the target function  $f(S)$  (probability or biochemical property) as  $f_R(R(S))$ , where  $R(S)$  is a trainable low-dimensional representation and  $f_R$  is smooth and trainable.

protein language models (*e.g.*, UniRep [14], ProGen [27], ESM-1b [28], ProVis [29], ProtTrans [30], and ProteinBERT [31]) are trained to reconstruct a sequence from a corrupted version with  $\sim 10$ – $20\%$  of the residues masked or randomly mutated, or to predict the next amino acid of a protein sequence given the previous ones. MSA context can also be provided (MSATransformer [32]). A general understanding of protein chemistry emerged from masked language modeling, such as similarities between amino acids, secondary structure elements, or tertiary contacts. The models can then be further fine-tuned to account for specifics of protein families, even for families with low number of sequences and/or diversity [14,33]. Madani et al. [33] used the ProGen2 language model, fine-tuned and conditioned on natural lysozyme families to design novel lysozymes with native-like function (66/90 of the designs) and/or low sequence identity to natural proteins ( $<40\%$ ). They further reported that both pre-training and fine-tuning were important for accurate activity prediction. In Ref. [34], Hie et al. used the ESM-1b language model to propose single-point mutants of various antiviral antibodies with reduced reconstruction error. After experimental characterization and recombination of the best mutants, they found that the binding affinity could be improved for 4/7 of the tested antibodies.

### Physics-inspired methods

The protein design problem amounts to finding a sequence with low free energy in prescribed conformational state(s) (as a monomer structure, in complex with ligands). Classically, this is achieved by (i) defining a tractable approximation of the free energy and (ii) minimizing it over the sequence. A common validation metric of design protocols is their sequence recovery rate: starting from the structure of a protein, its sequence-defined side chains are stripped, and a novel sequence is reconstructed from the remaining backbone atoms. The sequence recovery rate is the average sequence identity between the designed and original sequence: high values indicate that the structure-induced constraints on sequence are well recapitulated by the protocol.

Toward this end, a myriad of approximate physical force fields has been developed for protein design applications, including Rosetta [35,36] or FoldX [37]. These force fields are, however, heuristics that do not faithfully account for the underlying quantum dynamics and the ability of the sequence to effectively fold into said structure from an unfolded state. Moreover, evaluating the free energy further involves a thorough exploration of the conformation state beyond the target states. Limitations of force field-based protein design include high computational cost (slow and inefficient Monte Carlo-based optimization), unsatisfactory sequence recovery

rates from backbone structure (30–50%), and limited experimental success rate.

Recent progresses in machine learning-based structure prediction algorithms from sequence have opened the way to novel design paradigms. Deep learning models, such as RaptorX [38], AlphaFold1 [39], or trRosetta [40], predict various geometric features of protein structures from multiple sequence alignments, such as contacts, backbone dihedral angles, pairwise distance matrices, or angles between residue frames. Then, the model-computed negative log probability of the features of a conformation defines an effective potential that can be minimized to fold the protein. By analogy with the Gibbs–Boltzmann ensemble, this effective potential can be interpreted as the free energy of the folded state. This approximation bypasses the requirement to extensively sample the conformation space for free energy estimation [37]. Fixed-backbone design protocols based on trRosetta [37], AlphaFold2 [41], and language models [42] were recently proposed and experimentally validated. For binder design, binding free energy proxies can be similarly constructed: Gainza et al. [43,44] use a DL-computed matching score between molecular surface patches to design *de novo* mini-binding proteins.

What if the target conformation is partially or fully unspecified? Sequences with well-defined conformation can be similarly designed by minimizing the conformational entropy rather than the free energy. In these so-called hallucination protocols (initially introduced by *DeepDream* in the field of Computer Vision), the conformational entropy is quantified by the uncertainty of the structure prediction models. This is motivated here by the observation that low-confidence structure predictions often correspond to disordered regions of proteins [45]. Hallucination protocols based on trRosetta [46], AlphaFold2, and RoseTTAfold were recently proposed [47,48].

Following these approaches, several groups have successfully designed proteins with fully specified [49,50] or *de novo* backbone structures and constructed folds around functional motifs [47,51]. A potential limitation is sequence diversity and amino acid compositional bias. Indeed, amino acids with versatile conformations (*e.g.* multiple side-chain torsion angle) and thus inherently uncertain structures are disfavored in such confidence-maximization protocols. Thus, these design protocols may not encompass the full diversity of sequences that can effectively adopt a target fold. Another weakness of these protocols is the potential existence of “adversarial” optima: sequences with highly confident predictions for one model but not another, which “trick” the network rather than solve the design problem [52].

Alternatively, one can try to directly predict suitable sequences for a given fold, the so-called inverse folding problem. Unlike the folding problem, many sequences can adopt the exact same fold; hence, a distribution of sequences should be constructed. One approach is to “thread” a sequence along the target backbone, calculate a compatibility score between the sequence and the backbone, and iteratively mutate it to improve its score. Using a 3D-convolutional neural network, Anand *et al.* [53] predict possible amino-acid substitutions and corresponding rotamer states from current structure and iteratively mutate the protein to generate diverse sequences folding into a TIM-barrel. A second approach is to directly build a tractable distribution of sequences that can be easily sampled from: autoregressive generative models based on coarse-grained, graph-based representation of the protein backbone allow the generation of a full sequence in a single pass [54–59]. These models achieve higher sequence recovery rates and are much less computationally intensive than force field-based approaches. They have been proposed for fixed-backbone monomer design [60] as well as multimers [61] and antibody design [62,63]. Recently, Dauparas *et al.* [59] successfully designed various proteins using an autoregressive message-passing neural network.

Despite recent progress, these approaches still have some scope limitations: by construction, they are not suitable for modeling disordered proteins or segments. Fine-tuning of allosteric motion or catalytic activity remains a major challenge, as these models are trained on static structures and are coarse-grained. Another source of concern is that these models are increasingly diverging from physics: for instance, AlphaFold implicitly assumes the presence of molecular cofactors, post-translational modifications, or protein partners to properly fold a structure [64,65]. Thus, highly confident *in silico* predictions may prove false in experimental conditions, and the identification of model-derived matching scores to target physical properties is not always correct. For instance, some of the proteins designed by the trRosetta-based hallucination protocol [46] formed homo-oligomers or aggregates *in-vitro*, despite being predicted as monomers *in silico*.

### Synergistic methods

Evolutionary-based and physics-inspired approaches are highly complementary for both coverage and scope, and they have been used together extensively in classical protein design pipelines [5,6]. While physics-inspired models predict general biochemical properties (stability of the monomer and protein–ligand or protein–protein complexes), evolution-based methods learn various family-specific functional constraints in an agnostic fashion, including stability or catalytic activity, but also allosteric couplings [66] or specification of homo-oligomer state [67]. Thus, it is appealing to

combine both methods for optimal success rate. This can be achieved in multiple ways.

First, the evolutionary model can be used to rapidly generate diverse sequence libraries, and then, candidate sequences would be prioritized based on the scores obtained from more computationally intensive physics-inspired models. Examples include the PROSS and FUNCLIB web servers, which use Rosetta and PSSM information to automatically redesign enzymes toward increased stability or modified catalytic activity [68,69]. Tran *et al.* and Das *et al.* designed, respectively, cell-penetrating and antimicrobial peptides using generative models combined with molecular dynamics [70,71].

This simple approach is sufficient if a substantial fraction of evolutionary-designed sequences has satisfactory physical scores. Otherwise, multi-objective optimization/Monte Carlo sampling may be necessary to generate sequences that have both high evolutionary likelihood and physical score. The Rescue protocol redesigns sequences by optimizing a weighted sum of the Rosetta energy and the evolutionary score estimated by a Potts model [72].

An open question is whether separate physical and evolutionary models are necessary. Instead, could one learn physical interactions from evolution, and reciprocally, to predict evolution from structure? A promising direction is to train joint models of MSA and structure, adapted from language and structure prediction models [73,74]. Other options could include the fine-tuning of structure-based generative models using evolutionary information, or, conversely, the regularization of evolutionary models using structural information.

Should we always restrict the search space for solving a given design problem to the vicinity of a specific protein family? Indeed, recent design approaches based on pan-family, transformer-based sequence generative models [60], diffusion-based structure generative models [75], and joint sequence/structure diffusion-based generative models [66] enable search beyond a specific family. The latter showed impressive successes *in vitro* for monomer and binder design. If (i) the design problem is well-defined in terms of target properties (*e.g.* thermostability, binding affinity to a target,... etc.), (ii) the target properties can be faithfully approximated *in silico*, and (iii) efficient exploration algorithms exist, and then increasing the search space is a promising strategy. Otherwise, restricting the search space to the vicinity of a protein family is desirable, especially if the problem is ill-defined (*e.g.* finding a “human-like” protein with improved thermostability and catalytic efficiency for gene therapy [76], redesigning a yeast SH<sub>3</sub> domain while preserving its cellular protein interactions [20]).

## Summary and futures directions

In summary, evolutionary-based and physics-inspired approaches have undergone drastic improvement in the last years, enabling unprecedented success rates and democratization of protein design. However, important challenges remain.

Regarding evolutionary-based models, more efforts are required to develop models that can generalize among protein families, while maintaining computational tractability (for accessibility to the whole community), interpretability, and well-defined sampling protocols. Incorporation of known structural information as priors, for example, using structure-aware transformer models, such as EvoFormer [41] (where the structure is provided as template), is another interesting direction. Moreover, training protocols of evolutionary models should better account for phylogenetic relations between samples. In Ref. [77], Weinstein et al. argue that fitness prediction performances may plateau or decrease as model complexity increases without proper treatment of phylogeny—a phenomenon very recently observed with language models [78]. A conceptual and practical limitation is the entanglement of evolutionary constraints: current models do not have the ability to selectively discard specific constraints that are relevant *in vivo* but not for engineered proteins (*e.g.* requirement to bind inhibitor protein(s), adequacy to specific cellular compartment, etc.). Conversely, proteins that are evolutionary fitted at the family level may not be suitable for a specific biochemical task (*e.g.* they may bind a related ligand, but distinct from the prescribed one).

Physics-inspired methods have made impressive progress for the design of monomers and oligomers with fully or partially specified structure. In particular, spectacular improvements in success rates were reported for the *de novo* binder design problem [75,79], although subsequent *in vitro*-directed evolution remains the norm [43,74]. Altogether, finding sequences that adopt a well-defined conformation is becoming easier. On the other hand, modeling of flexible regions, such as immunoglobulin loops or peptides, catalytic sites (which require fine-grained description of intermediate catalytic states), or allosteric motions, remains challenging. Another key challenge is to better characterize the *in vivo* behavior of these designed proteins (*e.g.* off-target distribution of protein binders, lifetime, and immunogenicity). Future developments of ML-based molecular dynamics [80] and neural force fields docking algorithms, such as the recent DiffDock [81], could open up the way to a better understanding of these functions. Extensions to other types of ligands, such as ions, nucleotides, or small organic molecules, are also important future directions.

While synergistic approaches have been widely successful for non-ML design protocols [5,6], most recent ML-based studies considered either of the approaches. Yet, evolutionary-based and physics-inspired modeling are highly complementary, and combining them through simple proposal/acceptance or joint optimization protocols should lead to higher success rates for challenging design problems. In the longer run, models predicting MSAs from structures or, conversely, structure-based priors [82,83] for evolutionary models may allow overcoming the current limitations of each approach.

## Credit author statement

**C.M.:** Conceptualization, Investigation, Writing - Original Draft. **D.B., S.C., R.M.:** Conceptualization, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **J.T.:** Conceptualization, Investigation, Writing - Original Draft, Supervision, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

No data was used for the research described in the article.

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## References

Papers of particular interest, published within the period of review, have been highlighted as:

- \* of special interest
- \*\* of outstanding interest

1. The UniProt Consortium: **UniProt: a worldwide hub of protein knowledge**. *Nucleic Acids Res* 2019, **47**:D506–D515.
2. Bateman A, Birney E, Cerruti L, Durbin R, Ewinger L, Eddy SR, Griffiths-Jones S, Howe KL, Marshall M, Sonnhammer ELL: **The pfam protein families database**. *Nucleic Acids Res* 2002, **30**: 276–280.
3. Wu Z, Johnston KE, Arnold FH, Yang KK: **Protein sequence design with deep generative models**. *Curr Opin Chem Biol* 2021, **65**:18–27.
4. Ovchinnikov S, Huang P-S: **Structure-based protein design with deep learning**. *Curr Opin Chem Biol* 2021, **65**:136–144.
5. Weinstein J, Khersonsky O, Fleishman SJ: **Practically useful protein-design methods combining phylogenetic and atomistic calculations**. *Curr Opin Struct Biol* 2020, **63**:58–64.

6. Marques SM, Planas-Iglesias J, Damborsky J: **Web-based tools for computational enzyme design.** *Curr Opin Struct Biol* 2021, **69**:19–34.
  7. Eyre-Walker A, Keightley PD: **The distribution of fitness effects of new mutations.** *Nat Rev Genet* 2007, **8**:610–618.
  8. Weigt M, White RA, Szurmant H, Hoch JA, Hwa T: **Identification of direct residue contacts in protein–protein interaction by message passing.** *Proc Natl Acad Sci USA* 2009, **106**:67–72.
  9. Morcos F, Pagnani A, Lunt B, Bertolino A, Marks DS, Sander C, Zecchina R, Onuchic JN, Hwa T, Weigt M: **Direct-coupling analysis of residue coevolution captures native contacts across many protein families.** *Proc Natl Acad Sci USA* 2011, **108**:E1293–E1301.
  10. Trinquier J, Uguzzoni G, Pagnani A, Zamponi F, Weigt M: **Efficient generative modeling of protein sequences using simple autoregressive models.** *Nat Commun* 2021, **12**:5800.
  11. Russ WP, Figliuzzi M, Stocker C, Barrat-Charlaix P, Socolich M, Kast P, Hilvert D, Monasson R, Cocco S, Weigt M, *et al.*: **An evolution-based model for designing chorismate mutase enzymes.** *Science* 2020, **369**:440–445. 440.
- Evolution-based design.** Here, the authors trained a Direct Coupling Analysis generative model on a multiple sequence alignment of chorismate mutase enzymes, and used it to generate a diverse set of artificial sequences. A high fraction (83%) of designed sequences had native-like functionality, despite having limited sequence identity to known natural proteins.
12. Riesselman AJ, Ingraham JB, Marks DS: **Deep generative models of genetic variation capture the effects of mutations.** *Nat Methods* 2018, **15**:816–822.
  13. Tubiana J, Cocco S, Monasson R: **Learning protein constitutive motifs from sequence data.** *eLife* 2019, **8**, e39397.
  14. Alley EC, Khimulya G, Biswas S, AlQuraishi M, Church GM: **Unified rational protein engineering with sequence-based deep representation learning.** *Nat Methods* 2019, **16**: 1315–1322.
  15. Hawkins-Hooker A, Depardieu F, Baur S, Couairon G, Chen A, Bikard D: **Generating functional protein variants with variational autoencoders.** *PLoS Comput Biol* 2021, **17**, e1008736.
- Evolutionary-based design.** Here, the authors proposed regular and autoregressive variational autoencoders for generative modeling of protein sequences, and tested it for experimental design of luxA bacterial luciferase variants. They found that models trained on aligned rather than unaligned sequences were more successful at generating active sequences. In a second design iteration, they predicted solubility from learnt latent variables and used conditional sampling to generate soluble variants.
16. Repecka D, Jauniskis V, Karpus L, Rembeza E, Rokaitis I, Zrimec J, Poviloniene S, Laurynenas A, Viknander S, Abuajwa W, *et al.*: **Expanding functional protein sequence spaces using generative adversarial networks.** *Nat Mach Intell* 2021, **3**: 324–333.
  17. Shin J-E, Riesselman AJ, Kollasch AW, McMahon C, Simon E, Sander C, Manglik A, Kruse AC, Marks DS: **Protein design and variant prediction using autoregressive generative models.** *Nat Commun* 2021, **12**:2403.
  18. Luo S, Su Y, Peng X, Wang S, Peng J, Ma J: **Antigen-specific antibody design and optimization with diffusion-based generative models for protein structures.** 2022, <https://doi.org/10.1101/2022.07.10.499510>.
  19. Biswas S, Khimulya G, Alley EC, Esvelt KM, Church GM: **Low-N protein engineering with data-efficient deep learning.** *Nat Methods* 2021, **18**:389–396.
- Language/Evolutionary-based design.** Here, the authors propose and experimentally validate a design methodology based on UniRep, a protein language model and semi-supervised machine learning. They i) fine-tune the language model on the protein family of interest (green fluorescent proteins and beta-lactamases), ii) generate random mutants by error-prone PCR and experimentally determine functional activity iii) fit a machine learning surrogate model of functional activity using the latent representation of the language model as features iv) perform *in-silico* optimization of the activity and v) evaluate the novel designs.
20. Lian X, Praljak N, Subramanian SK, Wasinger S, Ranganathan R, Ferguson AL: **Deep learning-enabled design of synthetic orthologs of a signaling protein.** 2022, <https://doi.org/10.1101/2022.12.21.521443>.
  21. Figliuzzi M, Jacquier H, Schug A, Tenaillon O, Weigt M: **Coevolutionary landscape inference and the context-dependence of mutations in beta-lactamase TEM-1.** *Mol Biol Evol* 2016, **33**: 268–280.
  22. Bisardi M, Rodriguez-Rivas J, Zamponi F, Weigt M: **Modeling sequence-space exploration and emergence of epistatic signals in protein evolution.** *Mol Biol Evol* 2022, **39**, msab321.
  23. Weinstein EN, Amin AN, Grathwohl WS, Kassler D, Dissel J, Marks D: **Optimal design of stochastic DNA synthesis protocols based on generative sequence models.** In *Proceedings of the 25th international conference on artificial intelligence and statistics*. PMLR; 2022:7450–7482.
  24. Gioacchino AD, Procyk J, Molari M, Schreck JS, Zhou Y, Liu Y, Monasson R, Cocco S, Sulc P: **Generative and interpretable machine learning for aptamer design and analysis of *in vitro* sequence selection.** 2022, <https://doi.org/10.1101/2022.03.12.484094>.
  25. Mirdita M, von den Driesch L, Galiez C, Martin MJ, Söding J, Steinegger M: **Uniclust databases of clustered and deeply annotated protein sequences and alignments.** *Nucleic Acids Res* 2017, **45**:D170–D176.
  26. Steinegger M, Mirdita M, Söding J: **Protein-level assembly increases protein sequence recovery from metagenomic samples manifold.** *Nat Methods* 2019, **16**:603–606.
  27. Madani A, McCann B, Naik N, Keskar NS, Anand N, Eguchi RR, Huang P-S, Socher R: **ProGen: language Modeling for protein generation.** *ArXiv200403497 Cs Q-Bio Stat*; 2020.
  28. Rives A, Meier J, Sercu T, Goyal S, Lin Z, Liu J, Guo D, Ott M, Zitnick CL, Ma J: **Biological structure and function emerge from scaling unsupervised learning to 250 million protein sequences.** *Proc Natl Acad Sci USA* 2021:118.
  29. Vig J, Madani A, Varshney LR, Xiong C, Socher R, Rajani NF: **BERTology meets biology: interpreting attention in protein language models.** *arXiv*; 2021.
  30. Elnaggar A, Heinzinger M, Dallago C, Rehawi G, Wang Y, Jones L, Gibbs T, Feher T, Angerer C, Steinegger M, *et al.*: **ProtTrans: towards cracking the language of life code through self-supervised deep learning and high performance computing.** *IEEE Trans Pattern Anal Mach Intell* 2021, <https://doi.org/10.1109/TPAMI.2021.3095381>.
- Protein language modelling.** In this representative work on language models for proteins, the authors trained various transformer networks of increasing sizes using high-performance computing facilities, and demonstrated their ability to capture biophysical features. Transfer learning allowed prediction of various features such as cell localization, secondary structure, stability or ligand binding sites.
31. Brandes N, Ofer D, Peleg Y, Rappoport N, Linial M: **ProteinBERT: a universal deep-learning model of protein sequence and function.** *Bioinformatics* 2022, **38**:2102–2110.
  32. Rao RM, Liu J, Verkuil R, Meier J, Canny J, Abbeel P, Sercu T, Rives A: **MSA transformer.** In *Proceedings of the 38th international conference on machine learning*. PMLR; 2021:8844–8856.
- Language/Evolutionary model.** This work builds upon both recent language models and long-standing use of MSAs. Rather than a single-sequence, the MSA transformer takes as input a corrupted subset of the MSA, and aims at reconstructing the original one by intertwining attention operations between residues and between sequences. The MSA transformer outperformed single-sequence transformers and traditional DCA models for distant contact prediction, despite having much fewer parameters than the former (20M vs up to 6B) - suggesting a stronger generalization capability.
33. Madani A, Krause B, Greene ER, Subramanian S, Mohr BP, Holton JM, Olmos JL, Xiong C, Sun ZZ, Socher R, *et al.*: **Large language models generate functional protein sequences across diverse families.** *Nat Biotechnol* 2023, <https://doi.org/10.1038/s41587-022-01618-2>.
  34. Hie BL, Xu D, Shanker VR, Bruun TUJ, Weidenbacher PA, Tang S, Kim PS: **Efficient evolution of human antibodies from**

- general protein language models and sequence information alone.* 2022, <https://doi.org/10.1101/2022.04.10.487811>.
35. Rohl CA, Strauss CEM, Misura KMS, Baker D: **Protein structure prediction using Rosetta.** In *Methods in enzymology*. Academic Press; 2004:66–93.
  36. Lemay JK, Weitzner BD, Lewis SM, Adolf-Bryfogle J, Alam N, Alford RF, Aprahamian M, Baker D, Barlow KA, Barth P, et al.: **Macromolecular modeling and design in Rosetta: recent methods and frameworks.** *Nat Methods* 2020, **17**:665–680.
  37. Delgado J, Radusky LG, Cianferoni D, Serrano L: **FoldX 5.0: working with RNA, small molecules and a new graphical interface.** *Bioinformatics* 2019, **35**:4168–4169.
  38. Wang S, Sun S, Li Z, Zhang R, Xu J: **Accurate de novo prediction of protein contact map by ultra-deep learning model.** *PLoS Comput Biol* 2017, **13**, e1005324.
  39. Senior AW, Evans R, Jumper J, Kirkpatrick J, Sifre L, Green T, Qin C, Židek A, Nelson AWR, Bridgland A, et al.: **Improved protein structure prediction using potentials from deep learning.** *Nature* 2020, **577**:706–710.
  40. Du Z, Su H, Wang W, Ye L, Wei H, Peng Z, Anishchenko I, Baker D, Yang J: **The trRosetta server for fast and accurate protein structure prediction.** *Nat Protoc* 2021, **16**:5634–5651.
  41. Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Židek A, Potapenko A, et al.: **Highly accurate protein structure prediction with AlphaFold.** *Nature* 2021, **596**:583–589.
  42. Verkuil R, Kabeli O, Du Y, Wicky BI, Milles LF, Dauparas J, Baker D, Ovchinnikov S, Sercu T, Rives A: **Language models generalize beyond natural proteins.** *bioRxiv* 2022.
  43. Gainza P, Sverrisson F, Monti F, Rodolà E, Boscaini D, Bronstein MM, Correia BE: **Deciphering interaction fingerprints from protein molecular surfaces using geometric deep learning.** *Nat Methods* 2020, **17**:184–192.
  44. Gainza P, Wehrle S, Hall-Beauvais AV, Marchand A, Scheck A, Hartevelde Z, Ni D, Tan S, Sverrisson F, Goverde C, et al.: **De novo design of site-specific protein interactions with learned surface fingerprints.** 2022, <https://doi.org/10.1101/2022.06.16.496402>.
- Physics-inspired design** Here, the authors propose a novel approach based on surface-based geometric deep learning for the design of *de novo* mini-protein binders targeting specific sites along a target protein, and test it on four distinct targets. It consists in i) Identifying candidate binding sites along the protein with Masif-site, a surface-based protein binding site predictor ii) Fast screening of complementary helical motifs using Masif-seed, a surface-based interaction predictor iii) Scaffolding the helical motif using the Rosetta MotifGraft protocol and iv) experimental optimization of the binding affinity using site-directed mutagenesis and yeast display. In all four cases, they successfully designed binders with binding affinities in the nM range and with good agreement between predicted and experimentally-determined binding modes.
45. Porta-Pardo E, Ruiz-Serra V, Valentini S, Valencia A: **The structural coverage of the human proteome before and after AlphaFold.** *PLoS Comput Biol* 2022, **18**, e1009818.
  46. Anishchenko I, Pellock SJ, Chidyausiku TM, Ramelot TA, Ovchinnikov S, Hao J, Bafna K, Norn C, Kang A, Bera AK, et al.: **De novo protein design by deep network hallucination.** *Nature* 2021, **600**:547–552.
- Physics-inspired design** In this work, the authors generate a diverse set of sequences with unspecified but stable structures via “network hallucination,” *i.e.* a conformational entropy minimization scheme based on the trRosetta distogram predictor. About 20% of the designed sequences exhibited a stable fold and of those, the experimentally-determined structure matched well the predicted one. While no novel fold was discovered, hallucination can be used to efficiently scaffold binding motifs.
47. Wang J, Lisanza S, Juergens D, Tischer D, Watson JL, Castro KM, Ragotte R, Saragovi A, Milles LF, Baek M, et al.: **Scaffolding protein functional sites using deep learning.** *Science* 2022, **377**:387–394.
  48. Bryant P, Elofsson A: **EvoBind: in silico directed evolution of peptide binders with AlphaFold.** 2022, <https://doi.org/10.1101/2022.07.23.501214>.
  49. Jendrusch M, Korb J, Sadiq SK: **AlphaDesign: a de novo protein design framework based on AlphaFold.** 2021.
  50. Huang B, Xu Y, Hu X, Liu Y, Liao S, Zhang J, Huang C, Hong J, Chen Q, Liu H: **A backbone-centred energy function of neural networks for protein design.** *Nature* 2022, **602**:523–528.
  51. Tischer D, Lisanza S, Wang J, Dong R, Anishchenko I, Milles LF, Ovchinnikov S, Baker D: **Design of proteins presenting discontinuous functional sites using deep learning.** 2020, <https://doi.org/10.1101/2020.11.29.402743>.
  52. Moffat L, Kandathil SM, Jones DT: **Design in the dark: learning deep generative models for de novo protein design.** *bioRxiv* 2022.
- Synergistic design.** The authors leverage previously developed DARK framework (iterative expansion of the functional space starting from a few natural sequences) to propose a set of sequences that are further refined through AlphaFold and simulated annealing to match a targeted backbone structure
53. Anand N, Eguchi R, Mathews II, Perez CP, Derry A, Altman RB, Huang P-S: **Protein sequence design with a learned potential.** *Nat Commun* 2022, **13**:746.
  54. Ingraham J, Garg V, Barzilay R, Jaakkola T: **Generative models for graph-based protein design.** In *Advances in neural information processing systems*. Curran Associates, Inc.; 2019.
  55. Jing B, Eismann S, Suriana P, Townshend R, Dror R: **Learning from protein structure with geometric vector perceptrons.** *arXiv*; 2021.
  56. Jing B, Eismann S, Soni PN, Dror RO: **Equivariant graph neural networks for 3D macromolecular structure.** 2021, <https://doi.org/10.48550/arXiv.2106.03843>.
  57. McPartlon M, Lai B, Xu J: **A deep SE(3)-equivariant model for learning inverse protein folding.** 2022, <https://doi.org/10.1101/2022.04.15.488492>.
  58. Hsu C, Verkuil R, Liu J, Lin Z, Hie B, Sercu T, Lerer A, Rives A: **Learning inverse folding from millions of predicted structures.** 2022, <https://doi.org/10.1101/2022.04.10.487779>.
  59. Dauparas J, Anishchenko I, Bennett N, Bai H, Ragotte RJ, Milles LF, Wicky BIM, Courbet A, de Haas RJ, Bethel N, et al.: **Robust deep learning-based protein sequence design using ProteinMPNN.** *Science* 2022, **378**:49–56.
- Physics-inspired design.** In this recent representative work on structure-based sequence generative models, the authors develop ProteinMPNN, an autoregressive Message Passing Neural Network and extensively benchmark it *in vitro*. ProteinMPNN takes as input the backbone coordinates of a protein structure or complex - represented as a coarse-grained pairwise distance graph - and predicts its sequence in an autoregressive fashion. At inference time, the model can generate a diverse set of sequences in seconds, and is more accurate than the Rosetta fix\_bb design protocol. Conditional generation - *e.g.*, redesign of specific positions or of specific chains within a complex is also supported.
60. Gao Z, Tan C, Li SZ: **AlphaDesign: a graph protein design method and benchmark on AlphaFoldDB.** 2022.
  61. Anand N, Achim T: **Protein structure and sequence generation with equivariant denoising diffusion probabilistic models.** 2022.
  62. Jin W, Wohlwend J, Barzilay R, Jaakkola T: **Iterative refinement graph neural network for antibody sequence-structure Co-design.** *arXiv*; 2022.
  63. Schneider C, Buchanan A, Taddese B, Deane CM: **DLAB: deep learning methods for structure-based virtual screening of antibodies.** *Bioinformatics* 2022, **38**:377–383.
  64. Hekkelman ML, Vries I de, Joosten RP, Perrakis A: **AlphaFill: enriching the AlphaFold models with ligands and co-factors.** 2021, <https://doi.org/10.1101/2021.11.26.470110>.
  65. **AI revolutions in biology.** *EMBO Rep* 2021, **22**, e54046.
  66. Malinverni D, Marsili S, Barducci A, Rios PDL: **Large-scale conformational transitions and dimerization are encoded in the amino-acid sequences of Hsp70 chaperones.** *PLoS Comput Biol* 2015, **11**, e1004262.



67. Uguzzoni G, John Lovis S, Oteri F, Schug A, Szurmant H, Weigt M: **Large-scale identification of coevolution signals across homo-oligomeric protein interfaces by direct coupling analysis.** *Proc Natl Acad Sci USA* 2017, **114**:E2662–E2671.
68. Weinstein JJ, Goldenzweig A, Hoch S, Fleishman SJ: **PROSS 2: a new server for the design of stable and highly expressed protein variants.** *Bioinformatics* 2021, **37**:123–125.
69. Khersonsky O, Lipsh R, Avizemer Z, Ashani Y, Goldsmith M, Leader H, Dym O, Rogotner S, Trudeau DL, Prilusky J, *et al.*: **Automated design of efficient and functionally diverse enzyme repertoires.** *Mol Cell* 2018, **72**:178–186.e5.
70. Tran DP, Tada S, Yumoto A, Kitao A, Ito Y, Uzawa T, Tsuda K: **Using molecular dynamics simulations to prioritize and understand AI-generated cell penetrating peptides.** *Sci Rep* 2021, **11**:10630.
71. Das P, Sercu T, Wadhawan K, Padhi I, Gehrmann S, Cipcigan F, Chenthamarakshan V, Strobel H, dos Santos C, Chen P-Y, *et al.*: **Accelerated antimicrobial discovery via deep generative models and molecular dynamics simulations.** *Nat Biomed Eng* 2021, **5**:613–623.
- Synergistic design.** Here, the authors propose and test a synergistic multi-step filtering approach for designing antimicrobial peptides. It consists in i) Learning a general-purpose Wasserstein Autoencoder on all available short peptides from Uniprot ii) Training multiple biochemical property classifiers based on the latent space representation (antimicrobial activity, solubility, toxicity, stability) iii) Performing rejection-based sampling to generate peptides with desired properties iv) Refining selection by coarse-grained molecular dynamics of the peptide - membrane interaction. Two designed peptides exhibited promising broad-spectrum antimicrobial activity.
72. Schmitz S, Ertelt M, Merkl R, Meiler J: **Rosetta design with co-evolutionary information retains protein function.** *PLoS Comput Biol* 2021, **17**, e1008568.
- Synergistic design.** Here, the authors propose a fixed-backbone design protocol that jointly integrates evolutionary constraints via Direct Coupling Analysis and physical energy scores via Rosetta. Their protocol achieved a higher sequence recovery rate (70%) than protocols that do not include evolutionary information or only single-site frequencies.
73. Wang J, Lisanza S, Juergens D, Tischer D, Anishchenko I, Baek M, Watson JL, Chun JH, Milles LF, Dauparas J, *et al.*: **Deep learning methods for designing proteins scaffolding functional sites.** 2021.
74. Mansoor S, Baek M, Madan U, Horvitz E: **Toward more general embeddings for protein design: harnessing joint representations of sequence and structure.** *Biochemistry*; 2021.
75. Watson JL, Juergens D, Bennett NR, Trippe BL, Yim J, Eisenach HE, Ahern W, Borst AJ, Ragotte RJ, Milles LF, *et al.*: **Broadly applicable and accurate protein design by integrating structure prediction networks and diffusion generative models.** 2022, <https://doi.org/10.1101/2022.12.09.519842>.
76. Giessel A, Dousis A, Ravichandran K, Smith K, Sur S, McFadyen I, Zheng W, Licht S: **Therapeutic enzyme engineering using a generative neural network.** *Sci Rep* 2022, **12**:1536.
77. Weinstein EN, Amin AN, Frazer J, Marks DS: **Non-identifiability and the blessings of misspecification in models of molecular fitness and phylogeny.** 2022, <https://doi.org/10.1101/2022.01.29.478324>.
78. Nijkamp E, Ruffolo J, Weinstein EN, Naik N, Madani A: **ProGen2: exploring the boundaries of protein language models.** 2022.
79. Yao S, Moyer A, Zheng Y, Shen Y, Meng X, Yuan C, Zhao Y, Yao H, Baker D, Wu C: **De novo design and directed folding of disulfide-bridged peptide heterodimers.** *Nat Commun* 2022, **13**:1539.
80. Fu X, Xie T, Rebello NJ, Olsen BD, Jaakkola T: **Simulate time-integrated coarse-grained molecular dynamics with geometric machine learning.** 2022, <https://doi.org/10.48550/arXiv.2204.10348>.
81. Corso G, Stärk H, Jing B, Barzilay R, Jaakkola T: **DiffDock: diffusion steps, twists, and turns for.** *Molecular Docking* 2022, <https://doi.org/10.48550/arXiv.2210.01776>.
82. Ferruz N, Noske J, Höcker B: **Protlego: a Python package for the analysis and design of chimeric proteins.** *Bioinformatics* 2021, **37**:3182–3189.
83. Monzon V, Paysan-Lafosse T, Wood V, Bateman A: **Reciprocal best structure hits: using AlphaFold models to discover distant homologues.** 2022, <https://doi.org/10.1101/2022.07.04.498216>.