

# Prediction of amyloidogenicity based on the n-gram analysis

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## ABSTRACT

Amyloids are proteins associated with the number of clinical disorders (e.g., Alzheimer's, Creutzfeldt-Jakob's and Huntington's diseases). Despite their diversity, all amyloid proteins can undergo aggregation initiated by 6- to 15-residue segments, called hot spots. To find the patterns defining the hot-spots, we trained predictors of amyloidogenicity, using n-grams and random forest classifiers, based on data collected in the AmyLoad database. Only the most informative n-grams, selected by our Quick Permutation Test, were considered. Since the amyloidogenicity may not depend on the exact sequence of amino acids but on more general properties of amino acids, we tested 524,284 reduced amino acid alphabets of different lengths (three to six letters) to find the alphabet providing the best performance in cross-validation. The predictor based on this alphabet, called AmyloGram, was benchmarked against the most popular tools for the detection of amyloid peptides using an external data set and obtained the highest values of performance measures (AUC: 0.90, MCC: 0.63). Our results showed sequential patterns in the amyloids, which are strongly correlated with hydrophobicity, a tendency to form  $\beta$ -sheets and rigidity of amino acid residues. Among the most informative n-grams of AmyloGram we identified 15 that were already confirmed experimentally. AmyloGram is available as a web-server: [www.smorfland.uni.wroc.pl/amylogram/](http://www.smorfland.uni.wroc.pl/amylogram/). The code and results are publicly available at: [github.com/michbur/prediction\\_amyloidogenicity\\_ngram](https://github.com/michbur/prediction_amyloidogenicity_ngram).

Keywords: n-gram, amyloid, prediction, random forest, feature selection

## 1 INTRODUCTION

Amyloid aggregates have been observed in tissues of people suffering from neurodegenerative diseases, such as: Alzheimer's, Parkinson's, Huntington's and amyotrophic lateral sclerosis, as well as many other conditions (Vidal and Ghetti, 2011). These aggregates were also detected in disorders other than neurological, for example in diabetes of type 2 or certain types of a cataract. Cells in tissues with amyloid oligomers exhibit very high mortality. However, the exact mechanisms of the cytotoxicity have not been discovered. Amyloids are resistant to activity of proteolytic enzymes and chemical compounds due to the specific and highly ordered structure of their steric zipper. However, some strategies to prevent amyloid formation have been proposed, e.g. Härd and Lendel (2012).

The aggregation occurs when a cell environment fosters the partial unfolding of protein chains or their fragmentation in such a way that the parts prone to joining with similar protein fragments become exposed. The formation of the non-native partially unfolded conformation is required to start the aggregation, presumably by enabling specific intermolecular interactions including electrostatic attraction, hydrogen bonding and hydrophobic contacts (Chaturvedi et al., 2016).

Initially, the resulting molecules form clusters consisting of a few elements, which are called oligomers. Next, they grow into larger aggregates. The aggregation of proteins or their fragments may lead to amorphous (unstructured) clusters or amyloid (highly ordered) unbranched fibrils. Independently of the protein sequence and its original structure, aggregates always display a common cross- $\beta$  structure (Sawaya et al., 2007). The distinctive structure of the steric zipper enables the selective detection of amyloids from amorphous aggregates using either a variety of microscopic techniques or fluorescence of probes with which they form compounds.

Currently, it is believed that short peptide sequences of amyloidogenic properties, called hot spots, are responsible for the aggregation of amyloid proteins. Previous studies have suggested that amyloidogenic fragments may have regular characteristics, not only with regard to averaged physicochemical properties of their amino acids, but also the order of amino acids in the sequence.

It is important to distinguish between amyloidogenic and amyloid (or amyloidic) peptides, because only the former are capable of initiating the process of aggregation. The latter may consist of amyloidogenic hot-spots as well as other regions that are not directly responsible for the onset of aggregation process, although involved in the final aggregate. Several computational approaches have been proposed to model and predict both kinds of regions. Physics- and chemistry-based models used in FoldAmyloid (Garbuzynskiy et al., 2010) and PASTA2 (Walsh et al., 2014) utilize the density of the protein contact sites. Statistical approaches include production of frequency profiles, such as the WALTZ method (Maurer-Stroh et al., 2010) and machine learning methods, for example those developed in our group (Gasior and Kotulska, 2014) and a novel predictor APPNN based on neural networks (Família et al., 2015).

The aim of our study is to automatically generate thousands of created hot spot models, select from them the most appropriate one and from its analysis gain a new insight into the mechanism of amyloidogenicity. To do so, we combined n-gram analysis with the reduction of amino acid alphabet.

In bioinformatics, n-grams (k-mers) are continuous or discontinuous sequences of  $n$  elements. Employed as a feature extraction method, n-grams are widely used in various analyses of biological sequences. Our choice of n-grams was driven by their highly interpretable nature. This is a valuable feature because we are interested in identification of motifs that are most relevant to amyloidogenic properties of peptides.

Several studies highlighted that three-dimensional protein structure depends not only on the exact sequence of amino acids but also on their general physicochemical properties. Hence, a reduced amino acid alphabet (encoding), which represents certain subgroups of amino acids, can still retain the information about the protein folding (Murphy et al., 2000). Since amyloid aggregates, especially their hot spot regions, have very specific spatial organization, we investigated if these regions can be described by a shorter amino acid alphabet. Hence, we created multiple encodings based on the combinations of various physicochemical properties that might be associated with amyloidogenicity.

To discover amino acid patterns specific for amyloidogenicity, we based our analysis on n-grams, continuous or discontinuous sequences of length  $n$  drawn from the encoded peptides. The extraction of n-grams allows the detection of more elaborate motifs, but creates very large feature spaces. Henceforth, we used a novel feature selection algorithm, Quick Permutation Test (QuiPT), to select the most informative n-grams.

We used selected n-grams to train a predictor based on the random forest method (Breiman, 2001) to discriminate between amyloidogenic and non-amyloidogenic peptides. We trained the classifier for several iterations on peptides of varying lengths to identify the optimal number of residues which include the information about the occurrence or absence of a hot spot. In the cross-validation setup, we found the encoding associated with the best-performing classifier and its set of informative n-grams. Finally, we benchmarked our best-performing classifier, AmyloGram, on an external data set against state-of-the-art software tools for prediction of amyloid or amyloidogenic regions.

## 2 METHODS

### 2.1 Data set

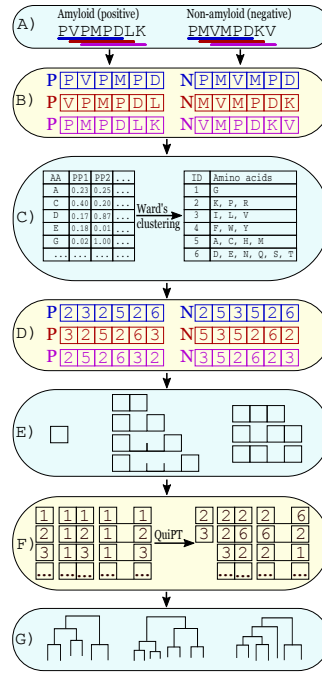
The data used in the study was extracted from AmyLoad data base (Wozniak and Kotulska, 2015). We obtained 418 amyloid peptides and 1039 non-amyloid peptides.

Sequences shorter than six and longer than 25 amino acid residues (i.e., 8 and 27 sequences, respectively) were removed from the set. The former were too short to be processed in the devised n-gram analysis framework and the latter were too diversified and rare, hampering the proper analysis.

In total, the final data set contained 1430 peptides: 397 amyloid and 1033 non-amyloid sequences.

### 2.2 Encodings of amino acids

The amyloidogenicity of a given peptide may not depend on the exact sequence of amino acids but on their more general properties. To verify this hypothesis, we handpicked 20 different measures from AAIndex data base (Kawashima et al., 2008) describing features important in the amyloidogenicity, such as: size of residues, hydrophobicity, solvent surface area, frequency in  $\beta$ -sheets and contactivity. We preferred more accurate measures introduced after 1980. The set of selected physicochemical properties was enriched by six measures representing amino acid contact site propensities Wozniak and Kotulska (2014). This gave us 26 features.



**Figure 1.** The scheme of n-gram extraction from studied peptide sequences. A) Source data: peptides with known amyloidicity status and indicated overlapping hexamers. B) Extraction of the overlapping hexamers with ascribed the amyloid status taken from their source peptide (P-positive, N - negative). C) Clusterization of amino acids into an encoding using a combination of various physicochemical properties (PP). D) Reduction of the amino acid alphabet in hexamers. E) Extraction of n-grams. From each hexamer, we extracted continuous n-grams with the length  $n = 1, 2$  or  $3$ . In addition, we selected gapped 2-grams with a gap of the length from  $1$  to  $3$  residues and gapped 3-grams with a single gap between the first and the second or the second and the third element of the n-gram. F) Selection of informative n-grams with Quick Permutation Test (QuiPT). G) Training of a random forest classifier using the n-grams selected in the previous step.

Since highly correlated measures would create very similar amino acid encodings, we further reduced the number of properties to 17 by selecting measures with the absolute value of Pearson's correlation coefficient smaller than 0.95 (Tab. 2).

Based on that, we created 524,284 encodings with different levels of amino acid alphabet reduction (three to six groups). Encodings were defined using Ward's clusterization (Joe H. Ward Jr, 1963), which was performed on all combinations of the normalized values of 17 selected physicochemical properties.

The majority of encodings had at least one duplicate. In such a case, only a single representative was included in the cross-validation. After filtering out the duplicates, we obtained 18,535 unique amino acid encodings.

We evaluated advantages of the proposed method for amino acids encoding by adding two standard encodings: (1) ADEGHKPNQRST, C, FY, ILMV, W (Kosiol et al., 2004) and (2) AG, C, DEKN-PQRST, FILMVWY, H (Melo and Marti-Renom, 2006), to check if the process of amyloidogenicity does require groupings different from more general amino acid classifications. We also added the full (unreduced) amino acid alphabet to evaluate potential benefits of the alphabet reduction.

### 2.3 Training sets

In the initial phase, we extracted overlapping hexamers from all peptides. Each hexamer was tagged with one of two etiquettes: amyloid (positive, i.e. originating from an amyloid peptide) or non-amyloid (negative, i.e. originating from a non-amyloid peptide). The etiquette ascribed to the hexamer was based on the amyloid propensity of its source peptide (Fig. 1 A and B). The hexapeptides constituted our training dataset.

Note that amyloid and non-amyloid elements of the set are not necessarily amyloidogenic or non-amyloidogenic, respectively. Hence, assuming that only a short part of the sequence in longer amyloids is responsible for amyloidogenicity, our method might result in many false positives in the training data set and in consequence yield inaccurate predictions as it was evaluated elsewhere

**Table 1.** Characteristics of training and test data sets used in the cross-validation. All sequences were derived from AmyLoad database. Training data sets are partially overlapping (e.g. set 6-10 contains also sequences from set 6). Test data sets are always disjointed.

Set	Sequence length	Status	Sequences	Hexamers
Training	6	Non-amyloid	841	841
		Amyloid	247	247
	6-10	Non-amyloid	964	1412
		Amyloid	312	475
	6-15	Non-amyloid	992	1653
		Amyloid	342	720
Test	6	Non-amyloid	841	841
		Amyloid	247	247
	7-10	Non-amyloid	123	571
		Amyloid	65	228
	11-15	Non-amyloid	28	241
		Amyloid	30	245
	16-25	Non-amyloid	41	571
		Amyloid	55	778

(Kotulska and Unold, 2013). To diminish this problem and facilitate the extraction of hot spots, we restricted the maximum length of peptides in the training data set to fifteen amino acids. This procedure should eliminate the problem of false negatives and reduce the number of false positives. Moreover, we expect that this influence of false positives would be naturally eliminated or significantly reduced from the pattern finally found in further steps of our method. On the other hand, allowing this ambiguity did not eliminate many hexamers of potentially high amyloidogenicity, whose propensity has not been experimentally proven.

To further study the problem of the length of the amyloidogenicity signal, we created three training sets with the sequences of varying lengths (Tab. 1). The smallest data set contained only the sequences of length 6. Assuming that the minimum length of the amyloidogenicity signal is the six residues, we can expect no false positive hexamers in this set. Moreover, we created two training sets with the progressively more liberal limit of the maximum sequence length (6-10 residues and 6-15 residues).

From each hexamer we extracted encoded n-grams (Fig. 1 D and E) with the length of 1, 2 and 3. In the case of 2- and 3-grams, we separately analyzed continuous and gapped n-grams. For 2-grams, we considered n-grams with the gap of the length from 1 to 3, whereas the 3-grams could contain a single gap between the first and the second or the second and the third position (see Fig. 1). The total number of n-grams depends on the the length of the encoding and is equal to 120, 260, 480 and 798 for encodings of length 3, 4, 5 and 6, respectively.

## 2.4 Quick Permutation Test (QuiPT)

The permutation tests are commonly used for filtering important n-grams testing hypothesis that an occurrence of n-gram and a value of a target are independent. However, they are computationally expensive and, as a result, they often become one of the most limiting factors in these kinds of analyses. Therefore, we developed the Quick Permutation Test which effectively filters n-gram features, without performing a huge number of permutations, using the information gain (mutual information) as the criterion of the importance of a specific n-gram. We used it to select the most discriminating n-grams extracted from the hexamers of the training data set. The counts of n-grams were binarized (1 if n-gram was present, 0 if absent). Only n-grams with the p-value smaller than 0.05 were assumed to be informative (Fig. 1 F).

Let us consider a contingency table for a target  $y$  and a feature  $x$ . For example, the entry  $n_{1,0}$  is the number of cases when the target is 1 and the feature is 0.

target / feature	1	0	total
1	$n_{1,1}$	$n_{1,0}$	$n_{1,\cdot}$
0	$n_{0,1}$	$n_{0,0}$	$n_{0,\cdot}$
total	$n_{\cdot,1}$	$n_{\cdot,0}$	$n$

Under the hypothesis that  $x$  and  $y$  are independent, the probability of observing such a contingency table is given by the multinomial distribution in which all probabilities depend only on marginal

**Table 2.** Selected 17 physicochemical properties used to create amino acid encodings.

Category	Property
Contactivity	Average flexibility indices (Bhaskaran and Ponnuswamy, 1988)
Contactivity	14 Å contact number (Nishikawa and Ooi, 1986)
Contactivity	Accessible surface area (Radzicka and Wolfenden, 1988)
Contactivity	Buriability (Zhou and Zhou, 2004)
Contactivity	Contact frequency in proteins from class $\beta$ , cutoff 12 Å, separation 5 Å (Wozniak and Kotulska, 2014)
Contactivity	Contact frequency in proteins from class $\beta$ , cutoff 12 Å, separation 15 Å (Wozniak and Kotulska, 2014)
$\beta$ -frequency	Average relative probability of inner beta-sheet (Kanehisa and Tsong, 1980)
$\beta$ -frequency	Relative frequency in $\beta$ -sheet (Prabhakaran, 1990)
$\beta$ -frequency	Thermodynamic $\beta$ -sheet propensity (Kim and Berg, 1993)
Hydrophobicity	Hydrophobicity index (Argos et al., 1982)
Hydrophobicity	Optimal matching hydrophobicity (Sweet and Eisenberg, 1983)
Hydrophobicity	Hydrophobicity-related index (Kidera et al., 1985)
Hydrophobicity	Scaled side chain hydrophobicity values (Black and Mould, 1991)
Polarity	Polarizability parameter (Charton and Charton, 1982)
Polarity	Mean polarity (Radzicka and Wolfenden, 1988)
Size	Average volumes of residues (Pontius et al., 1996)
Stability	Side-chain contribution to protein stability (kJ/mol) (Takano and Yutani, 2001)

distributions. The idea of the permutation test is to reshuffle labels of features and targets, while keeping the fixed total number of positives for features and targets. When we impose this constraint on the multinomial distribution, then the probability of occurrence for a given contingency table depends only on one entry, i.e.,  $n_{1,1}$ , which is fairly easy to compute. After computing Information Gain (IG) for each possible value of  $n_{1,1} \in [0, \min(n_{\cdot,1}; n_{1,\cdot})]$ , we get the distribution of Information Gain under the hypothesis that the target and feature are independent. We reject the null hypothesis of independence, when IG for the tested feature is above the required quantile from the IG distribution.

The analytic formula for the distribution enables to perform the permutation test much quicker. Furthermore, we get exact quantiles even for extreme tails of the distribution, which is not guaranteed by the random permutations. For example, for the test at the level  $\alpha = 10^{-8}$ , which can often occur in the corrections for multiple testing, the standard deviation of quantile estimate in the permutation test,  $\frac{p(1-p)}{m}$ , is roughly equal to  $\alpha$  itself even for a huge number of permutations like  $m = 10^8$ .

In the context of n-gram data, we can further speed up our algorithm. Note that test statistics depends only on  $n_{\cdot,1}$ , i.e., the number of positive cases in the feature when the target  $y$  is common for testing all n-gram features. Although we test millions of features, there are only a few distributions that we need to compute because the usual number of positives in n-gram feature is small. We take advantage of this fact and we compute quantiles only for the handful of distributions. Therefore complexity of our algorithm is roughly equal to  $O(n \cdot p)$  ( $n$  and  $p$  represents the number of features and number of positives, respectively).

Lastly, let us point out that QuiPT is very similar to Fisher's exact test. From the derivation provided in, e.g. (Lehmann and Romano, 2008), it becomes obvious that QuiPT is a heuristics for an unsolved problem of a two-tailed Fisher's exact test. In this heuristics, the extremity of a contingency table is defined by its information gain.

## 2.5 Cross-validation of encodings

The encoding yielding classifier with the best ability to correctly predict amyloidogenicity of peptides was chosen during the five-fold cross-validation. We used random forests as a method for classification and trained them on the binary n-gram data drawn from the overlapping hexamers, considering only n-grams selected by QuiPT (Fig. 1 G). We grown the forest keeping the default number of tree (500) and the default number of variables to possibly split in each node (the rounded down square root of the total number of variables). To speed up the computation, we used the fastest implementation of random forest in **R**, the ranger package (Wright and Ziegler, 2015).

A random forest separately considered all hexamers coming from a single peptide. If at least one hexamer extracted from a peptide was assessed as amyloidogenic, the whole sequence was denoted as amyloid. Otherwise, the peptide was classified as non-amyloid. Further, results were compared with the known etiquettes of the peptides to compute the performance measures.

Since a random assignment of peptides to subsamples in a cross-validation may result in the uneven number of hexamers in the subsamples (longer peptides yield more hexamers than shorter ones), we repeated the cross-validation fifteen times for each classifier to obtain more precise estimates of performance measures. We considered three length ranges of sequences in the training sets: 6, 6-10 and 6-15 residues, to evaluate if our classifiers are able to use decision rules extracted from sequences of a different length to correctly classify longer or shorter sequences.

To choose the most adequate amino acid encoding, we ranked the values of the Area Under the receiver operating characteristic Curve (AUC) for each particular classifier (assuming the rank 1 for the best AUC, rank 2 for the second best AUC and so on) and various ranges of the sequence length in the test data set. The encoding with the lowest sum of ranks from all sequence length categories was selected as the best one. For this encoding, we chose the range of the peptides length in the training set that provided the best AUC in the cross-validation.

## 2.6 Benchmark of AmyloGram

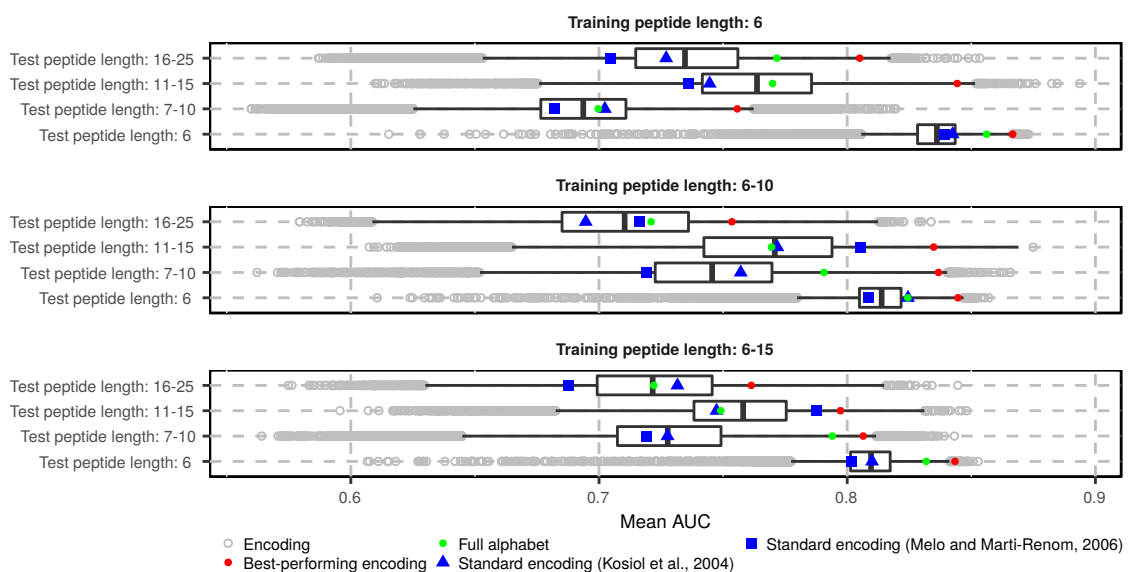
The best-performing encoding that had been chosen during the cross-validation of encodings was later used to train AmyloGram, n-gram based predictor of peptide amyloidogenicity.

To compare the performance of AmyloGram and other predictors of amyloids, we used external data set *pep424* (Walsh et al., 2014). Since some peptides were common for both *pep424* and AmyLoad, we removed them from the training data set. After the purification, the training data set for the benchmark consisted of 269 positive sequences and 746 negative sequences, all longer than five and shorter than fifteen residues. Aside from the removal of sequences, the training set of AmyloGram was identical to the training of classifiers during the cross-validation. The parameters of QuiPT and random forest algorithms were kept the same.

We removed peptides shorter than five amino acids from the *pep424* data set as our model of amyloidogenicity assumes the minimal length of six residues. Such change should not have affect the outcome of the comparison because only five sequences were eliminated (around 1% of the original data set). Beside the classifier based on the reduced amino acid alphabet, we also benchmarked three predictors based on the full 20-amino acid alphabet learned on n-grams extracted from sequences of different length ranges to separately assess the benefit of using only the n-gram analysis without the reduction of amino acid alphabet.

## 3 RESULTS AND DISCUSSION

### 3.1 Performance of the best encoding



**Figure 2.** Distribution of mean AUC values of classifiers with various encodings for every possible combination of training and testing data set including different lengths of sequences. The left and right hinges of boxes correspond to the 0.25 and 0.75 quartiles. The bar inside the box represents the median. The gray circles correspond to the encodings with the AUC outside the 0.95 confidence interval.



The n-grams occurring frequently in amyloids have often repeats of amino acids from the subgroup 3, suggesting that the presence of these amino acids in the vicinity might be one of the most effective predictors of amyloidogenicity. Hydrophobic and aromatic residues from the subgroup 4 are much less prevalent and never form repeats, but often co-occur with amino acids from the subgroup 3.

n-grams typical of non-amyloidogenic peptides have mostly one or more amino acids belonging to subgroups 2 or 6. These subgroups include strongly hydrophilic and highly flexible amino acids (K, P, R, D, E), which hamper the formation of  $\beta$ -structures. We observed that non-amyloidogenic n-grams usually contain more than one residue from one or two subgroups. Strong breakers of  $\beta$ -structures as K, P and R, belonging to the subgroup 2 are never present in amyloidogenic n-grams. In contrast, amino acids from subgroup 6 may rarely occur at the start or the end of such motifs, but are always balanced by a one or more hydrophobic residues.

Out of 65 the most informative n-grams, 15 (23%) were also found in the motifs validated experimentally for amyloidogenic and non-amyloidogenic peptides (López de la Paz and Serrano, 2004). The peptides used in this study are included in the AmyLoad data base, thus n-gram analysis is at least partially able to find the patterns in validated sequences.

### 3.3 Benchmark of AmyloGram

**Table 3.** Results of benchmark on *pep424* data set for PASTA2, FoldAmyloid, AmyloGram and its version learned on n-grams extracted for full amino acid alphabet from the sequences of the lengths specified in the brackets.

Classifier	AUC	MCC	Sensitivity	Specificity
AmyloGram (6)	0.8856	0.6057	0.6779	0.9037
full alphabet (6)	0.8411	0.5427	0.4966	<b>0.9593</b>
AmyloGram (6-10)	<b>0.8972</b>	<b>0.6307</b>	0.8658	0.7889
full alphabet (6-10)	0.8581	0.5698	0.7517	0.8259
AmyloGram (6-15)	0.8728	0.5420	<b>0.9463</b>	0.6111
full alphabet (6-15)	0.8610	0.5490	0.8188	0.7519
PASTA2	0.8550	0.4291	0.3826	0.9519
FoldAmyloid	0.7351	0.4526	0.7517	0.7185
APPNN	0.8343	0.5823	0.8859	0.7222

The benchmark covered AmyloGram as well as the three peer-reviewed predictors of amyloidogenicity: physical models included in PASTA2 (Walsh et al., 2014) and FoldAmyloid (Garbuzynski et al., 2010) as well as based on neural networks APPNN (Família et al., 2015). None of this methods is using reduced amino acid alphabet, but APPNN codes amino acids using the exact values of their physicochemical properties. Some known classifiers were not included in the benchmark because their performance on *pep424* data set is already known and lower than the performance of PASTA2 and FoldAmyloid (Walsh et al., 2014).

We analyzed AUC, Matthew's Correlation Coefficient (MCC), sensitivity and specificity (see Tab. 3). We used default settings for FoldAmyloid and APPNN. PASTA2 evaluated the input data in the 'Peptides' mode, which is advised by its authors for a peptide data.

Since PASTA2 does not return a probability of belonging to a specific category, we normalized the output data to compute the AUC values. The advised energy threshold (-5) was also normalized in the same manner and used as cut-off in computations of specificity, sensitivity and MCC. The resulting value of specificity 0.9519 is close to the value provided by its authors (0.95) and assures correctness of our computations. For other classifiers, including AmyloGram, we assumed a default 0.5 cut-off.

In the case of the studied data set, the n-gram extraction method appeared efficient enough to produce classifiers able to outperform other published methods. AmyloGram showed the highest AUC and MCC among all tested classifiers. It should be noted that it outperformed its counterparts trained on full amino acid alphabet. It is important to highlight that AmyloGram is the most balanced tool among all analyzed classifiers, having the best specificity/sensitivity trade-off, as indicated by the value of MCC.

The specificity of AmyloGram is lower than the specificity of PASTA2 but it is a consequence of the usage of the threshold value optimized for 0.95 specificity for the latter. If we assume for the AmyloGram the same threshold for the specificity, our classifier will still have a higher sensitivity (0.5518) than PASTA2. Therefore, if we assume such thresholds to both predictors, they will detect true non-amyloids with the same specificity but AmyloGram will predict more true amyloids.



Two of the three classifiers trained on n-grams using the full alphabet had also AUCs higher than PASTA2 and all three were more successful than FoldAmyloid, as well as APPNN. They also maintained the high specificity as seen previously during cross-validation. However, they generally performed worse than the classifier based on the reduced amino acid alphabet.

Among all considered predictors of amyloidogenicity, APPNN had the highest sensitivity. Nevertheless, its AUC was worse than AUCs of all n-gram-based predictors, as well as that of PASTA2 - indicating lower overall performance.

## 4 CONCLUSION

The description of peptides by short sub-sequences (n-grams) followed by the reduction of the amino acid alphabet allowed us to create the efficient predictor of amyloidogenic sequences called AmyloGram. One of the strengths of this approach is its highly interpretable outcome, because our methods provide explicitly short motifs relevant to amyloidogenicity of peptides and discriminating amyloids from non-amyloids. 65 important n-grams revealed that mostly aliphatic and nonpolar amino acids (isoleucine, leucine and valine), together with aromatic and also hydrophobic amino acids (phenylalanine, tyrosine, tryptophan) are good predictors of amyloid peptides.

Polar and hydrophilic residues from group 2 (K, P, R) never occur in n-grams associated with amyloidogenicity which is confirmed also by the experimental studies. On the contrary, residues from group 6 (D, E, N, Q, S, T), also polar, are present in both in amyloidogenic and non-amyloidogenic sequences. It seems plausible, that amino acids belonging to the subgroup 6 are necessary for the proper formation of some hot spots (hence their terminal position), but must be complemented by hydrophobic residues from the group 3 or 4. That means that hot spots are not completely hydrophobic and may contain a fraction of hydrophilic residues with the exclusion for known breakers of  $\beta$ -structures as lysine, proline and arginine.

Our studies confirm that the most important physicochemical properties associated with amyloidogenicity are hydrophobicity and tendency to forming  $\beta$ -sheets. We additionally discovered that amino acid flexibility can also sufficiently discriminate amyloid and non-amyloid peptides. The aggregating peptides tend to be characterized by more rigid chains. Most importantly, the Amylogram also showed sequential patterns of the amino acid groups appearing in the amyloids. Among the most informative n-grams we identified 15 that were already confirmed experimentally.

Our findings can be helpful in understanding the process of amyloid aggregation and recognition of peptides susceptible to the formation of amyloid aggregates involved in various diseases. Moreover, they might be employed in the creation of synthetic amyloid peptides. We anticipate that the described workflow is versatile enough to be applied in other areas of protein function prediction.

## FUNDING AND AVAILABILITY

Computations were carried out in Wrocław Center for Networking and Supercomputing (<http://www.wcss.pl>) and funded by the institutional grant No. 347. This research was partially funded by the KNOW Consortium and National Science Center (2015/17/N/NZ2/01845).

Our software is accessible as the web server under address [www.smorfland.uni.wroc.pl/amylogram/](http://www.smorfland.uni.wroc.pl/amylogram/). The web tool consists of AmyloGram (6-10). The threshold can be manually specified by a user. The code and results are publicly available at: [github.com/michbur/prediction\\_amyloidogenicity\\_ngram](https://github.com/michbur/prediction_amyloidogenicity_ngram).

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