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Prediction of Amphiphilic Cell-Penetrating Peptide Building Blocks from Protein-Derived Amino Acid Sequences for Engineering of Drug Delivery Nanoassemblies

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ABSTRACT

Amphiphilic molecules, forming self-assembled nanoarchitectures, are typically composed of hydrophobic and hydrophilic domains. Peptide amphiphiles can be designed from two, three or four building blocks imparting novel structural and functional properties and affinities for interaction with cellular membranes or intracellular organelles. Here we present a combined numerical approach to design amphiphilic peptide scaffolds that are derived from the human nuclear Ki-67 protein. Ki-67 acts, like a biosurfactant, as a steric and electrostatic charge barrier against the collapse of mitotic chromosomes. The proposed predictive design of new Ki-67 protein-derived amphiphilic aminoacid sequences exploits the computational outcomes of a set of web-accessible predictors, which are based on machine learning methods. The ensemble of such artificial intelligence algorithms, involving support vector machine (SVM), random forest (RF) classifiers and neural networks (NN), enables the nano-engineering of a broad range of innovative peptide materials for therapeutic delivery in various applications. Amphiphilic cell-penetrating peptides (CPP), derived from natural protein sequences, may spontaneously form self-assembled nanocarriers characterized by enhanced cellular uptake. Thanks to their inherent low immunogenicity, they may enable the safe delivery of therapeutic molecules across the biological barriers.

Introduction

The development of novel drug delivery systems to improve the bioavailability of therapeutic compounds and reduce severe side effects is a research area of growing interest.¹⁻²⁴ Drug delivery nano-assemblies are composed of either lipids, peptides, surfactants, carbohydrates or polymers besides of different combinations of multiple amphiphilic ingredients.²⁵⁻³⁹ Screening of new carrier structures by application of machine learning in materials science⁴⁰⁻⁴⁹ creates alternatives to the complex synthesis of new drug molecules, which require a long and expensive process of biological assays and physico-chemical characterization of the formulations up to clinical trials.

The focus of the present work is on bio-inspired peptides with self-assembly properties as designed candidates for more efficient and safe drug delivery nanocarriers (Figure 1). Cell penetrating peptides (CPP) are short sequences of amino acids (generally up to 35 aa), which have a capacity to cross the cellular bilayer membranes without damaging them.⁵⁰⁻⁶⁰ The research on CPPs has been inspired by the fact that proteins (like transcription-activating factors, signal sequences and homeoproteins) involve subunits that may facilitate their translocation across the plasma membranes into the inner cellular compartments.^{50-52,61-63} This finding has stimulated a number of investigations on the design of synthetic aminoacid sequences, which self-assemble and enable the cellular internalization of various compounds.^{3-7,53-60,64-84} The large diversity of CPPs has been classified into sequences of cationic, anionic or neutral nature.⁵⁰ CPPs can be either protein-derived^{53,73} or synthetic ones.^{56,72} Other CPP classifications regard their amphipathic character and their conformation type (*e.g.* α -helical, β -sheet or other).^{50-52,75} CPPs may transport and deliver different types of bioactive molecules into living cells (anticancer drugs, RNA, DNA, or proteins).^{3-7,35-39,54,57-60} It is expected that CPPs derived from native

proteins and characterized by amphiphilic nature may be more efficient as drug delivery systems thanks to their self-assembly properties and low immunogenicity.

Figure 1. *Top panel:* Self-assembled nanostructures formed by amphiphilic peptides: (A) vesicles, (B) micelles, (C) nanotubes, and (D) rod-like objects exemplifying precursors of fibers. *Bottom panel:* Molecular architecture of an amphiphilic peptide composed of hydrophilic (blue) and hydrophobic (yellow) domains. The red dots indicate positively charged residues along the aminoacid sequence. The latter can be a cell penetrating peptide (CPP).

When immersed in aqueous media, amphiphilic molecules (lipids, surfactants, peptides, lipid-drug or peptide-drug conjugates, *etc.*) self-assemble through a spontaneous process leading to the formation of organized supramolecular structures.⁶⁴⁻⁸⁴ Figure 1 shows a few examples of nanoscale self-assembled architectures of amphiphilic derivatives. The underlying self-assembly mechanism is governed by hydrophobic interactions, dipole and electrostatic forces, π - π stacking, and hydrogen bonding.⁷⁵ In a first approximation, the shapes of the generated self-assembled aggregates are determined, according to the Israelachvili's concept, by the hydrophobic/hydrophilic balance and the sizes of the hydrophilic and hydrophobic domains of the molecules.^{66-68,75,76} However, a same chemical composition may yield different supramolecular aggregates depending on the environmental conditions (*e.g.* pH, salt type and concentration, temperature, *etc.*).^{30,33,70,77,80} The resulting topologies may comprise vesicles, spherical or elongated micelles, tubules and donut shapes of nanoscale dimensions as examples (Fig. 1). The shapes, which may globally span in space, correspond to indefinite nanotubes, fibers, and gels. The properties of the obtained amphiphilic nanostructures can be tuned by the inclusion of various functionalities towards the design of nano-biomaterials for a wide range of biomedical applications (*e.g.* vaccines, drug delivery, diagnostic imaging, tissue engineering and regeneration).^{4-7,26,27,33-39}

The present work exploits chosen computational approaches, developed on the basis of machine learning, for the design of amphiphilic peptides suitable for drug delivery applications. We first compare the published predictors of CPPs, some of which make use of artificial intelligence algorithms based on support vector machine (SVM) and random forest (RF) classifier methods.⁸⁵⁻¹⁰² We then apply selected web-accessible programs to the prediction of new peptide amphiphiles from the sequence of the human protein Ki-67 (as a protein of interest).

The combined results of the validated methods for CPP and transmembrane helix prediction will yield peptide sequences with natural biocompatibility and targeting properties, which are valuable characteristics for potential therapeutic applications. Based on such numerical computations, the research on amphiphilic peptides can be expanded to assist the design of safe and efficient nanocarriers for biomolecular delivery and specific targeting. In further steps, the stability of the predicted peptide scaffolds can be increased by the inclusion of unnatural amino acid residues. Various building blocks can be integrated towards functionalization of the peptides, for instance signal domains for improved selectivity, lipid anchors for enhanced amphiphilicity, vaccine epitopes, or fluorescent labels for theranostic purposes.^{3,13-15,26,27,37,84}

Methods

Choice between rational and machine learning assisted strategies for design of cell-penetrating peptides and amphiphilic derivatives thereof

The CPP class of peptides has gained significant interest since the finding that the HIV-1-derived Tat peptide sequence can cross the cellular membranes to trans-activate the viral promoter.^{61,62} Some of the first CPPs have been found serendipitously by studying the internalization of chemically synthesized peptides, which have been derived from the third helix of the homeodomain of *Antennapedia*.⁶³

Rational design of biomimetic cell-penetrating peptides has been performed by Karagiannis *et al.*,⁵⁵ Stupp *et al.*,^{76,78} and Zhang *et al.*⁷¹ among others.^{56,72,75,80} Peptide amphiphile structures consisting of up to four building blocks (a hydrophobic alkyl chain, a β -sheet forming segment, a bioactive epitope and charged groups) have been phenomenologically designed with the purpose to achieve both self-assembly and functional features of the generated constructs.⁷⁶ Molecular

dynamics (MD) simulations of self-assembled peptide systems have been performed by different groups.⁸² It has been experimentally shown that the hydrophobicity plays a crucial role for the shape of the resulting self-assembled aggregates.⁹ The structures produced with synthetic peptides, such as K3-(WL)3-W-NH₂), C₁₆-WAAAAKAAAAKAAAAK), or C₁₈-KTTKS, have been in agreement with the molecular shape concept of the critical packing parameter, which determines the type of the obtained aggregates (micelles, vesicles or nanotube rods).^{66-68,71,72,83,84} Some studies have yielded ambiguous conclusions about the topology of the self-assembled peptide scaffolds because the Israelachvili's prediction does not account for the intermolecular interactions between α -helical moieties nor for the hydrogen bonding interactions between peptides of β -sheet conformations.^{4,13,34,75,80}

The initially proposed numerical approaches for CPP prediction have mainly been based on trial and error methods.^{57,58} These time-consuming analyses have permitted the constitution of a database of cell-penetrating peptides,^{46,85} but could not satisfy the growing market demand yet. The existing challenge has led to the emergence of an entirely new way for the prediction of aminoacid sequences based on numerical methods.⁸⁵⁻¹⁰² The first generation of the *in silico* algorithms has been dedicated to CPPs predictions based on quantitative structure-activity relationship (QSAR) studies.⁸⁵ This method determines the score of a peptide according to the physico-chemical properties of its amino acid sequence. Such features have been calculated by Sandberg and colleagues through the principal component analysis (PCA) algorithm.⁸⁵ The considered descriptors, referred to as Z-factors, are related to the polarity, molecular weight, and orbital calculations. This approach has turned out to be simplistic because it has not taken into account the order of the amino acid residues in the studied peptide sequences. However, the obtained results have been essentially valuable as the method predicted about 95% of the known

CPPs to have cell-penetration characteristics. At the same time, only 68% of the known non-CPPs sequences have been predicted to be such. The limitation of this approach has been associated with the fact that the dataset used to train the algorithm has been composed of a small number of peptides. The latter have been extracted from heterogeneous experimental studies using different experimental parameters (concentrations, types of cells, *etc.*). Furthermore, it has been established that the training process of the QSAR method was biased, which may strongly affect the results of the predictions.

Subsequently, a new generation of algorithms came out with the development of artificial intelligence.⁸⁶⁻⁹² The first one has been based on the support vector machine classifier (SVM) and quantitative structure-activity relationship models.⁸⁶ The SVM involves information about the protein sequence, a position-specific aminoacid propensity, and physicochemical properties. The classifier has achieved a maximal accuracy of 83% by excluding the difficult aminoacid sequences. Sanders *et al.* have overcome this problem and have emphasized the importance of using balanced datasets of peptides.⁸⁷ The SVM classifier has been applied to a benchmark dataset using a total of 145 peptides, 111 of which were known CPPs and the others were known non-CPPs. Subsequently, the SVM classifier method has been used by Gautam *et al.* who have extended the benchmark dataset to a much larger set consisting of 708 peptides.⁸⁸ For this CellPPD predictor, the percentage of accuracy has increased to 81% on the independent datasets.

The machine training on neuronal network (NN) has emerged later and has become publicly available by the algorithm CPPpred conceived by Holton *et al.*⁸⁹ The accomplishment of this approach has revealed that the accuracy of the algorithm increases when the redundancy of the training sequences is reduced by 80% (despite of the small training set of CPPs).

Random Forest (RF) classifier is another machine learning approach to predict peptides with cell penetrating properties.⁹⁰ The RF algorithm integrates a number of decision trees and chooses the classification with the highest number of votes from the trees. The RF predictor is trained on various physicochemical and biochemical properties of amino acids and pairs of amino acids such as amino acid frequency, electrostatic charge, molecular volume, polarity, and secondary structure. In the random subsets of variables, each tree depends on the values of a random vector, which is sampled independently with the same distribution for all trees in the forest. A majority voting scheme is then applied for the classification purposes.

We have chosen the neural network (CPPpred) and the support vector machine classifier (CellPPD) algorithms for the prediction of novel CPP sequences from the nuclear protein Ki-67. These CPPs will be merged to hydrophobic signal sequences or membrane spanning fragments in order to form amphiphilic peptide scaffolds. The web-accessible predictor Skip-CPPpred, which is based on a random forest (RF) classification,⁹¹ will also be included in the employed here algorithm set.

Biological small-angle X-ray scattering (BioSAXS) for characterization of amphiphilic peptide nanoassemblies and particles

Biological small-angle X-ray scattering (BioSAXS), which comprises solution X-ray scattering, is an indispensable tool for the characterization of biological macromolecules and their propensity for self-assembly in aqueous media.¹⁰³⁻¹⁰⁸ Amphiphilic peptide assemblies represent randomly oriented supramolecular aggregates in a solution phase. The degree of arrangement and disorder leads to characteristic patterns when the samples are exposed to the X-ray beam. The scattering emerges upon interaction between the X-ray photons and the electrons

in the studied samples. Thus, the BioSAXS method provides ensemble-averaged results about the peptide molecules or particles dispersed in the aqueous medium.¹⁰³⁻¹⁰⁸

In aqueous environment, self-assembly of peptide amphiphiles into nanoscale aggregates occurs above a critical concentration. Structural evolution and transitions between different particle topologies are possible at increasing peptide concentrations. To probe the topologies of the peptide structural ensembles, the experimental SAXS profiles should be compared with computed theoretical scattering profiles. Such models have been developed for solutions of monodispersed identical particles with minimal interactions in a dispersed state.¹⁰⁹⁻¹¹² The derived analytical expressions of the scattering profiles provide information about the shape of the nanoscale objects through the form factors.¹⁰⁹

Particle form factors for various shape models can be considered in the analysis of the SAXS patterns, for instance monodispersed and homogeneous solid spheres, spherical shells, monodisperse cylinders, rods, discs, ellipsoids, cylinders with an elliptical cross section, or core/shell structures. The analytical models, employed in the particle shape analysis, describe the relationship between the scattered intensity and the particle shape for monodisperse particle solutions. Equations for form factors of different geometrical objects are used for this purpose.¹⁰⁹⁻¹¹¹

Exemplary SAXS plots of model functions, which simulate the scattering of nanoparticles of ideal geometrical shapes,¹¹² are presented in Figure 2 for selected values of the variable parameters.

(A) Spherical particles (solid spheres) are simulated by a model scattering function with a single parameter, *i.e.* the sphere radius (50 nm);

(B) Hollow spherical particles (vesicles) are described by a model scattering function with two parameters, namely the radius of the spherical shell (50 nm) and thickness of the lipid bilayer shell (3.5 nm);

(C) Hollow cylinders (nanotubes) are anisotropic particles, the scattering of which is simulated by three parameters, *i.e.* the length of the tube (300 nm), the radius of the cylindrical shell (50 nm) and the thickness of the lipid layer shell (3.5 nm);

(D) Nonflexible cylindrical rods (fibers) are simulated by two-parameter model scattering function – the length of the fiber (300 nm) and its radius (50 nm).

Figure 2. Examples of computed small-angle X-ray scattering (SAXS) patterns of monodispersed self-assembled amphiphilic peptide objects. The presented SAXS plots comprise

form factor scattering from a sphere (A), a hollow sphere (vesicle) (B), a hollow circular cylinder (nanotube) (C), and a rigid rod (fiber) (D).

BioSAXS experiments were performed at the SWING beamline of synchrotron SOLEIL (Saint Aubin, France). The samples were sealed in capillaries for X-ray measurements. The q -vector was defined as $q = (4\pi/\lambda) \sin\theta$, where 2θ is the scattering angle. The synchrotron radiation wavelength was $\lambda = 1.033 \text{ \AA}$. The synchrotron SAXS patterns were recorded with a two-dimensional EigerX 4-M detector (Dectris, Baden, Switzerland) at 12 keV. The exposure time was 1 s. No radiation damage was observed at this exposure time. An average of three spectra per capillary was acquired. Scattering patterns of an empty capillary and a capillary filled with MilliQ water were recorded for intensity background subtraction.

The small angle X-ray scattering (SAXS) data processing included fitting of the experimental scattering intensities by a library of analytical expressions available in the SASfit software package for SAXS analysis.¹¹²

Results

Design of new cell-penetrating peptides and application of the chosen set of algorithms for the prediction of amphiphilic aminoacid scaffolds from protein-derived sequences

The design of novel biocompatible CPP building blocks is inspired here by the features of the naturally occurring protein Ki-67.¹¹³⁻¹¹⁵ This human protein is composed of 3241 amino acids (see Fig. 3 below). It is an important nuclear protein that has been suggested to function as a

steric and electrostatic charge barrier against the aggregation of mitotic chromosomes.¹¹³ The cellular localization of the Ki-67 protein is a cell-cycle-phase dependent.

The performed peptide design takes into account that the spontaneous self-assembly process of protein-derived peptides is controlled by the hydrophobic/hydrophilic balance of the aminoacid constructs. The tendency for assembly into micelles, nanotubes, vesicles, nanofibers, nanobelts, or nanosheets in aqueous solutions will depend on the composition and the relative sizes of the two constituting building blocks (Fig. 1). The latter will impact the organization of the final aggregates. If the length of the hydrophobic domain is too long, the peptide solubility will be low. As a consequence, the corresponding critical aggregation concentration (CAC) might be too low for the development of drug delivery carriers. A sufficiently high concentration of positively charged amino acids in the hydrophilic domain can stabilize the thermodynamic equilibrium of the dispersed supramolecular aggregates (Fig. 1). Moreover, it can provide a higher affinity for interaction with the negatively charged cellular membranes, which is essential for efficient intracellular delivery of biomolecules.

For the selection of candidate peptides with a membrane insertion potential, aminoacid sequences must be identified from protein domains that reside or interact with lipid plasma membranes. Transmembrane helices¹¹⁶ can be derived from the muscarinic acetylcholine receptor M2, the angiotensine 1A receptor, the metabotropic glutamate receptor, or the human galanin receptor type 1 as examples. Peptides with high membrane affinity involve also magainin 2, melittin, α -synuclein, orexin-A, lactoferricin, indolicidin, eledoisin, and endothelin-1 among others.^{10,58,74} The hydrophobic scaffold domains, derived from transmembrane spanning segments, will facilitate the membrane fusion, translocation, or endocytosis of the created peptides.

Our purpose here is to build, by the chosen computational tools, bio-inspired amphiphilic peptides comprising two distinct building blocks: a hydrophilic domain and a hydrophobic domain (Fig. 1, bottom panel). The protein-derived hydrophilic domain should involve positively charged amino acid fragments from the Ki-67 sequence, whereas the hydrophobic domain should be mainly composed of non-polar amino acids. The properties of the constructed protein-derived peptides will be computed and optimized by the cluster of web-accessible predictor software presented in Table 1.

Table 1. Machine learning based algorithms and web-based prediction tools employed for the calculation of the peptide properties.

Name	Principal	Outcomes
EMBOSS	Associates charges to amino acids and performs an average	Charge distribution of a protein sequence
I-Tasser	Hierarchical approach using different algorithms (Iterative Threading ASSEmbly Refinement)	Protein structure and function predictions; secondary structure prediction
CellPPD	Support vector machine (SVM)	CPP score
CPP pred	Neuronal Network (NN)	CPP score
SkipCPP-Pred	Random forest (RF) classifier	CPP score
TMHMM	Hidden Markov Model	Transmembrane helix prediction

Towards the identification of hydrophilic aminoacid sequences with cell penetrating properties, derived from the human antigen protein Ki-67, we started with the evaluation of its charge distribution using the EMBOSS system.¹¹⁷ Figure 3 (top panel) shows the localization of the largest positively charged aminoacid cluster identified along the sequence of the Ki-67 protein (Fig. 3, bottom panel).

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1  MWPTRRLVTI  KRSGVDGPHF  PLSLSTCLFG  RGIECDIRIQ  LPVVSQHQCK  IEIHEQEAIL
61  HNFSSNTPTQ  VNGSVIDEPV  RLKHGDUVIT  IDRSFRYENE  SLQNGRKSTE  FPRKIRBQEP
121  ARRVSRSSF  SDPDEKAQDS  KAYSKITTEGK  VSGNPQVHIK  NVKEDSTADD  SKDSVVAQGT
181  NVHSSEHACR  NCRNAADPIS  GDFKEISSVK  LVSRYGELKS  VPTTQCLDNS  KKNESPFWKL
241  YESVKKELDV  KSKKENVLQY  CRKSGLQTDY  ATEKESADGL  QGETQLLVSR  KSRPKSGGSG
301  HAVAEPASPE  QELDQNKGKG  RUVESVQTPS  KAVGASFPY  EPAKMKTPVQ  YSQQQNSPQK
361  HKNKDLTYTG  RRESVNLGKS  EGFKAGDKTL  TPRKLSLRNR  TPAKVEDAAD  SATKPENLSS
421  KTRGSIPTDV  EVLPTETETI  NEFFLTWLTL  QVERKIQKDS  LSKPEKLGTT  AGQMCSSGLP
481  LSSVDINNFG  DSIINESEGI  LKRRRVSFGG  HLRPELFDEN  LPNTPPLKRG  EAPT KRRSLV
541  MHTPPVLKKI  IKEQPPSGK  QESGSEIHVE  VKAQSLVIS  PAPSRRKTPV  ASDQ KRRSCK
601  TAPASSSKSQ  TEVPRGGRK  SGNLPSKRVS  ISRSQHDILQ  MICS KRRSGA  SEANLIVAKS
661  WADVVKLGAK  QTQTKVIKKG  PQRSMNKROR  RPAATPKKPV  EVHSQFSTGH  ANSPCTIIIG
721  KAHTEKVHVP  ARPYRVLNNF  ISNQKMDTKE  DLSGIAEMFK  TPVKEQPQLT  STCHIAISNS
781  ENLLGKQFQG  TDSGEELLP  TSESGGNVF  FSAQNAARQP  SDKCSASPLL  RRQCIRENGN
841  VAKTPRNTYK  MTSLETKTS  TETEPSKTVS  TANRSRSTSE  FRNIQKLPVE  SKSEETNTEI
901  VECILKRGQK  ATLLQQRREG  EMKEIERPFE  TYKENIELKE  NDEKMKAMKR  SRTWQKCAP
961  MSDLTDLKSL  PDTELMKDTA  RQNLQLQTQD  HAKAPKSEK  KITKMPQSL  QPEINTPTH
1021  TQQLKASLG  KVGVKEE  LLA  VGFTRTSGE  THTHRE  PAG  DGK  SIRT  FKE  SP  QILD  PAA
1081  RVTGMKKWPR  TPKEEAQSL  E  DLAGE  KE  LFG  TP  GP  SE  SMT  DE  KT  TK  IACK  SP  PPE  SVD  TP
1141  TSTKQWPKRS  LRKADVEEF  L  LAL  RK  LT  PSA  GK  AM  LTP  KPA  GG  DE  KD  IKA  F  MG  TP  VQ  KLD  L
1201  AGTLPQSKRQ  LQTPKEKQA  A  LED  LAG  FKE  L  FQ  TP  GH  TEE  L  VA  AG  KT  TK  IP  CD  SP  QSD  PVD
1261  TPTSTKORPK  RSIRKADVEG  E  LLAC  RN  IMP  SAG  KAM  HTP  K  PS  VG  EE  KD  II  IF  VG  TP  VQ  KL
1321  DLTENLTGS  KRRPQTPKEEA  Q  ALED  LT  GF  K  EL  FQ  TP  GH  TE  EA  VA  AG  KT  TK  MP  CE  SS  PPE
1381  ADPTPTSTRQ  PKTPLEKRDV  Q  KE  LS  AL  KKL  TQ  TS  GE  TT  HT  DK  VP  GG  ED  KS  IN  AF  RE  TA  KQ
1441  KLDPAASVTG  SKRHPTKKEK  A  QPLE  DL  AGL  KE  LF  QTP  VCT  DK  PT  THE  KTT  KI  AC  RS  QDP
1501  VDTPTSSKPQ  SKRSLRKVDV  E  EEF  FAL  KKG  TP  SAG  KAM  HT  PK  PAV  SGE  KN  IY  AF  MG  TP  VQ
1561  KLDLTENLTG  SKRELQTPKE  K  QALED  LAG  FKE  LF  QTP  BGH  TE  SM  TND  KT  AK  VAC  KSS  QP
1621  DPDKNPASSK  RRLKTSLGKV  G  VKEE  LL  AV  G  KL  TQ  TS  GE  TT  HT  HE  PT  GD  G  KSM  KAF  MES  P
1681  KQILDASAASL  TGSKRQLRTP  K  KSE  VP  ED  L  AG  FIE  LF  QTP  SH  TK  ES  MT  NE  KT  TK  VS  YRAS
1741  QPDLDVTPTS  SKPQPKRSLR  K  AD  TEE  E  FLA  FR  HQT  PS  AG  K  AM  HT  PK  PAV  G  EE  KD  INT  FL  G
1801  TPVQKLDQPG  NLPGSNRRLL  L  TRKE  KA  QALE  EL  TG  FE  RF  Q  TP  CT  DN  PT  TD  EK  TT  KK  IL  CK
1861  SPQSDPADTP  TNTKORPKRS  L  K  KAD  VEE  EF  LA  FR  KL  TP  PSA  GK  AM  HT  PK  AA  VGE  KD  INT  F
1921  RETAKKLDL  LGNLPGSKR  R  PQTPKEKAKA  LED  LAG  FKE  L  FQ  TP  GH  TEE  S  MT  DD  KI  TE  VS
1981  CKSPQDPVVK  TPTSSKORLK  I  SLG  KV  GVK  E  EV  LP  VG  KL  TQ  TS  CK  TT  QTH  R  ET  AG  DG  KS  IK
2041  AFKESAKQML  DPANYGTGME  R  WPT  TP  KEE  A  QS  LED  LAG  FKE  EL  FQ  TP  DH  TE  EST  TD  DK  TT  K
2101  IACKSPPPES  MDTPTSTRR  R  PKT  PLG  KR  DI  VE  EL  S  AL  KQL  TQ  TT  HT  DK  VP  QED  KG  IN  VF
2161  RETAKKLDL  AASVTGSKRQ  P  RT  PK  CK  AQ  P  LED  LAG  LKE  L  FQ  TP  ICT  DK  P  TTE  KT  TK  IA
2221  CKSPQDPVVK  TPTIFKPKSK  R  SLR  KAD  VEE  ES  LAL  KKT  P  SV  GK  AM  DT  PK  PAG  GDE  KDM  K
2281  AFMGTPVQKL  DLPGNLPGSK  R  WP  QT  PKE  KA  QALED  LAG  FKE  EL  FQ  TP  GT  DK  PT  DE  KT  TK  I
2341  ACKSPQDPV  DTPASTKORP  R  KRN  LR  KAD  VE  EE  FLA  KKT  P  PS  AG  KAM  DT  P  KPA  VS  DE  KNI
2401  NTFVETPVQK  LDLLGNLPGS  K  KRP  QTP  KE  K  AE  ALED  LV  GF  KE  LF  QTP  GH  T  EE  SM  TD  DK  IT
2461  EVSCKSPQPE  SFKTSRSSKQ  R  LK  IP  LV  KVD  MKEE  PL  AV  SK  LT  RT  SGE  TT  Q  TH  TE  PT  GDS  K
2521  SIAFKESPK  QILDPAASVT  G  SR  ROL  R  TR  K  EK  A  R  ALED  LV  DF  KE  LF  SAP  G  HT  EE  SM  TID  K
2581  NTKIPCKSP  PELTDATST  T  KRC  PK  TR  PR  K  EV  KE  EL  SAV  E  RL  TQ  TS  QST  H  TH  KE  PAS  GD
2641  EGIKVLROR  KKKPNPVEEE  P  SR  RR  PR  AP  K  E  KA  QPLE  DL  GF  TE  L  SET  SG  HT  QE  SL  TACK
2701  ATKIPCESPP  LEVVDTTAST  T  KR  HL  RT  RV  QK  VQ  VK  EEP  SAV  KF  TQ  TS  GE  TT  DAD  KE  PAG  ED
2761  KGIKALKESA  KQTPAPAASV  T  CS  RR  R  PR  AP  K  E  SA  QAI  ED  L  AG  FK  DP  AAG  H  TE  SM  TD  DK  T
2821  TKIPCKSSPE  LEDTATSKR  R  PR  TR  AQ  KVE  VK  EE  LL  AV  GK  LT  QT  SGE  TT  TH  TD  KE  PV  GE  GK
2881  GTKAFKQPAK  RKLDAEDVIG  S  RR  QP  RAP  KE  KA  QPLE  LAS  FQ  EL  SQ  TP  GH  TE  EL  AN  GA  D
2941  SFTSAPKQTP  DSGKPLKISR  R  VL  RAP  KVE  P  VGD  VV  STR  DP  VK  SQ  SK  SNT  S  LP  PL  PF  KR  GG
3001  GKGGSVTGTR  RLRCMPAPEE  I  VE  EL  P  AS  KK  Q  R  VA  PR  AR  CK  SSE  PV  VIM  KR  SL  RT  SAK  RIE
3061  PAELNSNDM  KTNKEEHLQ  L  DSV  PEN  KG  IS  LR  SR  R  ON  KTE  AE  QQ  I  TE  VF  V  LA  ER  IE  IN  RN
3121  EKKPMKTSPE  MDIQNPDDGA  R  KP  IP  RD  KVT  EN  KR  CL  RS  AR  Q  NE  SS  QP  KVA  EE  SC  QK  SAK
3181  VIMQCKGKG  EAGNSDSMCL  R  SR  RT  KS  QPA  AS  TLE  SK  SVQ  RV  TR  SV  KR  CA  EN  PK  KA  ED  NV
3241  CVKKIRTRSH  RDSER

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Figure 3. *Top panel:* Charge distribution along the aminoacid sequence of the protein antigen Ki-67 represented by EMBOSS.¹¹⁷ The observed maximum is associated with the presence of a cluster (KRRR) of positively charged residues. The human protein Ki-67 (composed of 3241 amino acids) is encoded by the MKI67 gene. *Bottom panel:* Amino acid sequence of the human

Ki-67 protein with an indication of positively charged aminoacids (lysine [K] or arginine [R], yellow colour) and the clusters of charged residues (red and green colours).

Computational scans were realized with the chosen natural protein sequence (Fig. 3, bottom panel) using the set of algorithms listed in Table 1. This allowed identifying the regions of aminoacid residues that correspond to the positively charged fragments (as determined by the calculated charge distribution in Fig. 3). Then, a second scan of the algorithmic approach was realized in order to extract the potential cell-penetrating peptide (CPP) sequences inside the Ki-67 protein. Two algorithms were used for this purpose: CellCPP and CPPpred (Table 1). Based on the obtained scores, the most promising peptide fragments, each of 10aa length, were chosen for the constitution of the hydrophilic domain of the designed peptides.

Selected results of the performance of the machine learning prediction methods are presented in Table 2. The new cell penetrating peptides, which we derived from the biosurfactant protein Ki-67, are indicated in blue color as sequences of 10 amino acids (Table 2). Towards the objective to design novel amphiphilic peptides as candidate drug delivery carriers, we decided to merge the obtained new cell penetrating peptides (*e.g.* KKKARQKLVK) with chosen hydrophobic fragments, which may enable the targeting of the lipid plasma membranes or the intracellular membranes. Candidate hydrophobic chains of aminoacid residues were selected from the protein databank of transmembrane proteins (pdhtm).¹¹⁶ Interesting fragment sequences were chosen based on the criteria of simplicity, secondary structure, and length. Examples of such hydrophobic domain sequences, *e.g.* the mitochondria-targeting N-terminal region of the hexokinase-II protein and the membrane spanning segment of the acetylcholine receptor, are shown in Table 2 (yellow color).

Table 2. Examples of numerically predicted amphiphilic peptides and their properties calculated by the set of algorithms summarized in Table 1. The positively charged hydrophilic fragments are indicated in blue and the hydrophobic domains are indicated in yellow. Representative CPPpred and SkipCPP-pred scores are obtained for all cell penetrating peptides derived from the human protein Ki-67.

	Peptide amino acid sequence	Length (aa)	CPPpred score	SkipCPP-pred score	Transmembrane helix score
1:	KKKARQKL VK NLE TF FY ALL HS AIM	25	0.5	0.58	0
2:	GSRRRPRAP RM STAISV LLAQA V FLL TS Q	29	0.6	0.87	0.6
3:	GSRRRPRAP TA ISV LLAQA V FLL TS Q	27	0.75	0.89	0.2
4:	KRRP RT RA QK MSTAISV LLAQA V FLL TS Q	29	0.7	0.81	0.6

Practically, several amphipathic peptides were created by joining hydrophilic and hydrophobic moieties in all possible configurations. The resulting various combinations were numerically examined by the ensemble of algorithms (Table 1) for the prediction of their cell penetrating and transmembrane helix behaviour. The peptide chains were examined by I-Tasser (for the secondary structure and solvent accessibility), TMHMM (for the transmembrane probability rate and hydropathy), and CPPpred and SkipCPP-pred for the determination of the CPP scores (Table 2). The molecular models of the predicted peptide architectures with lengths of 25-29 residues long are shown in Figure 4.

Figure 4. Molecular models of the numerically predicted amphiphilic peptide sequences given in Table 2. The cell penetrating peptide fragments are newly derived from the human nuclear protein Ki-67.

Discussion

Contribution of SAXS to establish the amphiphilic peptide self-assembly

Structural results obtained by SAXS revealed that the peptide amphiphile building blocks, predicted by the numerical approaches and yielding significant CPPpred and SkipCPP-pred scores, may easily self-assemble into nanoscale objects above a critical aggregation concentration. The SAXS data obtained from solutions of the predicted peptides were analyzed using analytical expressions restricted to simple shapes and geometries. An ellipsoidal shape of

the self-assembled aggregates was deduced from the analysis of the synchrotron small-angle scattering (BioSAXS) data shown in Figure 5. The performed fitting of the experimental scattering intensities by a model scattering function from a population of ellipsoids of revolution yielded the best outcome. The investigated amphiphilic peptide MIASHLLAYFFTELNKVLKQRAKKK has been designed with the purpose to target the mitochondria. Liu *et al.* have shown that the critical aggregation concentration of the peptide can be decreased by anchoring of a long fatty acid chain to the hydrophobic terminus of the aminoacid sequence MIASHLLAYFFTELN.⁹ This effect has enhanced the bioactivity of peptide conjugate upon mitochondrial targeting of lung cancer cells.⁹

Figure 5. Synchrotron small-angle scattering (BioSAXS) pattern presenting an experimental evidence for the self-assembly propensity of the predicted amphiphilic peptide MIASHLLAYFFTELNKVLKQRAKKK in an aqueous medium. The peptide construct consists of the mitochondria-targeting N-terminal region of the protein hexokinase-II

(MIASHLLAYFFTELN) and a cell penetrating peptide derived from the Ki-67 sequence (*i.e.* KVLKQRAKKK).

The shapes and the sizes of the self-assembled amphiphilic peptide aggregates are crucial for the drug delivery efficacy in addition to their charge and interfacial characteristics. The transport and diffusion properties of nanocarriers with elongated shapes are different from those of the sphere-shaped particles. The latter move faster and accumulate to a lower extent at interfaces. At variance, the circulation time of the rod-like particles is prolonged with regards to spherical ones. Thus, the nanocarrier shape and geometry have impact on the drug transport across the biological barriers and the cellular internalization process.

Elongated, rod- and tubule-shaped nanoparticles may be more efficient in controlled release applications because of the increased circulation time and the promoted adhesive interactions with the cellular membranes. However, it is challenging to achieve a control over their asymmetric geometries in the nanoscale range. Moreover, such long objects may cause remodeling of the plasma membranes.

Quite often, the self-assembly of amphiphilic peptides yields fibers, which considerably exceed nanoscale dimensions and form micrometer-long aggregates.^{7,76} Long nanotubes may also reach macroscopic sizes. Delivery carriers of such micrometer-large dimensions are challenging for a systemic administration of drugs and generally should be avoided for parenteral use (via injections). Consequently, topical applications, such as implants and intracutaneous administration, have been envisioned for their clinical use.⁷

Drug delivery carriers of sizes typically below 100 nm can evade the systemic clearance. Therefore, the ellipsoid-like nanoassemblies, as those reported in Figure 5, may be expected to display higher therapeutic efficacy in parenteral drug delivery.

Discussion on the applications of amphiphilic peptides in drug delivery

The advantages of using peptides as delivery systems rather than other chemicals rely on their biocompatibility, their low cost and facile synthesis using standard solid-phase techniques, their tunable bioactivity, and targeting opportunities including for instance nuclear localization sequences (NLS) or mitochondria targeting fragments. The peptide self-assembly into nanostructures can provide reservoirs with enhanced drug delivery efficiency and can permit the extension of the half-life of the encapsulated biomolecules. Peptide-based drug delivery carriers comprise various supramolecular architectures including nanotubes, vesicles, micelles, cubosomes, and hydrogels constituted of self-assembled fibers.^{4,6,7,13,15,24,26,33-35} Vesicles and micelles offer interesting possibilities to encapsulate or covalently bind therapeutic molecules.^{20,22,26,27,76-78}

The research on application of amphiphilic peptides in therapeutic delivery and regenerative medicine (*e.g.* ocular drug delivery, vaccine delivery, and anticancer agents delivery) constantly increases.^{3-10,13-24,35-39} Besides the wide range of potential biomedical applications in tissue engineering, imaging and diagnostics, comprehensive reviews have been published about the use of amphiphilic peptides for delivery of biotherapeutics such as oligonucleotides, proteins, or siRNA.^{3,7,23,24,26,35,37,51,52,58,75-84} Cell-penetrating peptide amphiphiles can form complexes with siRNAs and mediate the delivery of nucleic acids toward gene silencing in gene therapy.^{35,53-58} They have successfully delivered drugs and photosensitisers *in vitro* and *in vivo*, for instance in

cultured rabbit lens epithelial cells.¹⁹ The reported uptake mechanisms are varied among endocytosis, membrane breaking and translocation, or macropinocytosis.

One of the most promising research directions on peptide amphiphiles includes cancer treatment strategies.^{5,20-22} Recent reports have shown that treatments of breast cancer could be improved by coupling the active drug molecule to a cell-penetrating peptide.^{20,37,39} The conjugation of an anti-cancer cargo to a CPP can improve tumor targeting and the *in vivo* drug transport, thus reducing the side effects of the therapy.^{20,39}

Peptide-based hydrogels have been used for controlled drug release and are the most common and studied self-assembled peptide nanostructures at the time.^{6,7,67,78,84} The regenerative potential of hydrogels composed of peptide amphiphile nanofibers has been examined in various applications, *e.g.* traumatic injury of spinal cord, bone regeneration, and promotion of neurite formation.^{67,79} Peptide amphiphile hydrogels have provided sustained release of therapeutic proteins (*e.g.* trophic factors) in topical applications.⁷ The bioactivity of the entrapped proteins has been preserved over long periods. Hydrogels of supramolecular peptide amphiphile nanofibers, loaded with drugs and high affinity antibodies, can suppress tumor growth in cancer therapy. A concrete example is the RADA16-I peptide,¹⁵ which has been the subject of a number of studies on protein release kinetics, breast tissue reconstruction, and also for the elaboration of peptide fibrous scaffolds for commercial purposes (*e.g.* the *PuraMatrix*TM product).

Conclusion

Taking into account the expanding applications of amphiphilic peptides in nanobiomaterials science and regenerative nanomedicine, the development of efficient algorithms for CPPs

prediction and evaluation of the peptide properties is crucial for the acceleration of the translational research into clinics.

We proposed a combined computational design approach, which comprises a set of algorithms that is applied for scanning of naturally occurring protein sequences for CPP fragments towards the identification of candidate amphiphilic peptides with desired features. The amphiphilic peptide scaffolds are created from at least two building blocks. The hydrophobic domains can be designed from transmembrane protein fragments, which are available in the protein databank of transmembrane proteins (pdbtm). The hydrophilic domains can be designed as CPP sequences, which are derived from proteins with biosurfactant properties. In perspective, the outcome of the suggested combined algorithm may include also the uptake score of the CPPs and their mode of cellular internalization.

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