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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 cellpenetrating 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 immerged 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, $\pi - \pi$ stacking, and hydrogen bonding.⁷⁵ In a first approximation, the shapes of the generated selfassembled 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 cellpenetrating peptides and amphiphilic derivatives thereof

The CPP class of peptides has gained significant interest since the finding that the HIV-1derived 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-WAAAAKAAAAKAAAAKA), 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);

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(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 2 θ is the scattering angle. The synchrotron radiation wavelength was $\lambda = 1.033$ Å. 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 metabotrobic 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	
ТМНММ	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).



1	MWPTRRLVTI	KRSGVDGPHF	PLSLSTCLFG	RGIECDIRIO	LPVVS <mark>KO</mark> HCK	IE IHEQEAIL
61	HNESSTNPTO	VNGSVIDEPV	RLKHGDVITI	ID <mark>R</mark> SF <mark>R</mark> YENE	SLONG <mark>RK</mark> STE	FP <mark>RK</mark> I <mark>REQE</mark> P
121	ARRVSRSSFS	SDPDEKAODS	KAYSKITEGK	VSCNPOVHIK	NVKEDSTADD	S <mark>K</mark> DSVAQGTT
181	NVHSSEHAGR	NGRNAADPIS	GDFKEISSVK	LVS <mark>R</mark> YGE L <mark>K</mark> S	VPTTQC LDNS	KKNE SPFWKL
241	YESVKKELDV	KSOKENVLOY	CRKSGLOTDY	ATEKESADGL	QGETQLLVSR	KSRPKSGGSG
301	HAVAE PAS PE	QELDONKCKG	RUVESVQTPS	KAVGASFPLY	EPAKMKTPVQ	YSQQQNSPQ <mark>K</mark>
361	HKNKD LYTTG	RRESVNLGKS	EGF <mark>K</mark> AGD <mark>K</mark> TL	TPRKLSTRNR	TPA <mark>K</mark> VEDAAD	SAT <mark>K</mark> PENLSS
421	KTRGS IPT DV	EVLPTETEIH	NEPFLTLWLT	OVERKIOKDS	LS <mark>K</mark> PEKLGTT	AGCMCSGLPG
481	LSSVDINNFG	DSINESEGIP	LKRRRVSFGG	HLRPELFDEN	LP PNTP L <mark>KR</mark> G	EAPTKRKSLV
541	MHT PPVLKKI	IKEOPOPSCK	QESGSEIHVE	VKAQSLVISP	PAPSPRETPV	ASDORRESCK
601	TAPAS SSKSQ	TEVPKRGGRK	SCINLPSKRVS	ISRSQHDILQ	MICSKRRSGA	SEANLIVA <mark>K</mark> S
661	WADVVKLGAK	OTOTKVIKHG	POR SMNKROR	RPAT PKK PVG	EVHSOFSTCH	ANSPCTIIIG
721	KAHTE KVHVP	A <mark>R</mark> PY <mark>R</mark> VLNNF	ISNQ <mark>K</mark> MDF <mark>K</mark> E	DLSGIAEMFK	TPVKEQPQLT	STCHIAISNS
781	ENLLGROFOG	TDSCEEPLLP	TSESFGGNVF	FSAONAAROP	SDKCSASPPL	RROCIRENCN
841	VAKTPRNTYK	MTS LET <mark>K</mark> TSD	TETEPSKTVS	TANRSCRSTE	FRNIOKLPVE	S <mark>K</mark> SEETNTEI
901	VECILKRGOK	ATLLOORREG	EMKEIERPFE	TY <mark>KENIE L</mark> KE	NDEKMKAMKR	S <mark>R</mark> TWGO <mark>K</mark> CAP
961	MSDLTDLKSL	PDTELMKDTA	ROONLLOTOD	HAKAPKSEKG	KITKMPCQSL	QPEPINTPTH
1021	TROOL KAS LG	KVGVKEE LLA	VG <mark>K</mark> FT <mark>R</mark> TSGE	TTHTHRE PAG	DGKSIRTFKE	SP <mark>K</mark> DILDPAA
1081	RVTOMKKWPR	TPKEEAQSLE	DLAGE <mark>K</mark> E LFQ	TPGPSEE SMT	DEKTTKIACK	SPPPESVDTP
1141	TST <mark>KOWPKR</mark> S	LRKADVEEEF	LALRKLTPSA	G <mark>K</mark> AMLTP <mark>K</mark> PA	GGDEKDIKAF	MGTPVQKLDL
1201	AGT LPGSKRQ	LOTPKEKAQA	LED LAGE <mark>K</mark> E L	FQTPGHTEE L	VAAG <mark>KTTK</mark> IP	CDSPQSDPVD
1261	TPTSTKORPK	RSIRKADVEG	ELLACENIMP	SACKAMHTPK	PSVGEEKDII	IFVGTPVQKL
1321	DLTENLTGS	REPOTPKEEA	OALEDLTGEK	ELFOTPGHTE	EAVAAGKTTK	MPCESSPPES
1381	ADTPTSTRRQ	PKTPLEKRDV	OKELSALKKL	TOTSCETTHT	DKVPGGEDKS	INAFRETARO
1441	KLDPAASVTG	SKRHPKTKEK	AOPLEDLAGL	KE LEOTPVCT	DKPTTHEKTT	KIACRSOPDP
1501	VDTPTSSKPQ	SKRSLRKVDV	EKEFFAL	TPSAGKAMHT	PKPAVSGERN	IYAFMGTPVQ
1561	KLDLTENLTG	SKRELOTPKE	KADALEDLAG	FRELFOTRCH	TEESMINDKT	AKVACKSSOP
1621	DPDKNPASSK	RELETSLORV	GVKEELLAVG	KLIQTSGETT	HTHTEPTGDG	KSMKAPMESP
1681	KUI LUSAASL	TGSKRULKTP	KGKSEVPEDL	AGFIELEUTP FDFDFDCACK	SHTKESMINE	KTTKVSIKAS
1901	TRACKLOOPC	NLDCSNRRLO	TREELLA	FUTCERFLEO	TECTENET	FRTTRETLCE
1061	C DOCD DAD TO		INCERADABE	INFORTEDED		VCEENDINE
1921	VCTIVERLDI.	LONLDCS	DOLDKEKYXX	LEDLACEREL	ROTDCHTERS	MUDDRITTEVS
1991	CKSDODDDVK	TTTSSKORLK	TSLCKVCVKF	FVLDVCKLTO	TSCKTTOTHE	FTACDCKSTK
2041	AFKESAKOMI.	DPANYCTOME	RWDRTPKEEA	OSLEDLACEK	RLEOTPOHTE	ESTTDDETT
21.01	TACKSPPPES	MOTPTSTERR	PRTPLOKEDT	VEELSAL	TOTTHTOKVP	CDEDRCTNVF
21.61	RETAKOKLDP	AASVTGSKRO	PRTPKCKAOP	LEDLAGLKEL	FOTPICTDKP	TTHERTTRIA
2221	CRSPOPDPVG	TPTTERPOSK	RSLRKADVEE	ESLAL	SVCKAMDTPK	PACCOEKDMK
2281	AFMCTPVOKL	DLPCNLPGSK	RWPOTPKEKA	OALEDLAGEK	ELFOTPGTDK	PTTDEKTTKI
2341	ACKSPOPDPV	DTPASTKORP	KENLEKADVE	EEFLALRERT	PSAGKAMDTP	KPAVSDEKNI
2401	NTEVETPVOK	LDLLGNLPGS	KROPOTPKEK	AEALEDLVGF	KE LFOT PGHT	EESMTDDKIT
2461	EVSCKSPOPE	SEKTSRSSKO	RLKIPLVKVD	MKEEPLAVSK	LTRTSGETTO	THTEPTCDSK
2521	SIKAFKESPK	OILDPAASVT	GSRROLRTRK	EKARALEDLV	DFKE LFSAPG	HTEE SMTIDK
2581	NTKIPCKSPP	PELTDTATST	KRCPKTRPRK	EVKEELSAVE	RLTOTSCOST	HTHKEPASCO
2641	EGIKVLKORA	KKK PN PVEEE	PSRRRPRAPK	EKAOPLEDLA	GFTE LSET SG	HTOESLTACK
2701	ATKIPCESPP	LEVVDTTAST	KRHLR TRVOK	VOV <mark>K</mark> EEPSAV	KETQTSCETT	DAD <mark>K</mark> EPAGED
2761	KGI KALKESA	KOTPAPAASV	TGSERRPRAP	RESAQAIEDL	AGFKDPAAGH	TEESMTDDKT
2821	TKIPCKSSPE	LEDTATS SKR	RPR TRAOKVE	VKEE LLAVGK	LTQTSGETTH	TD <mark>KEPVGEG</mark> K
2881	GT <mark>KAF KO</mark> PAK	RKLDAEDVIG	SRROPRAPKE	KAOP LED LAS	FOELSOTPCH	TEELANGAAD
2941	SFTSAPKOTP	DSGKPLKISR	RVLRAPKVE P	VGDVVSTRDP	VKSQSKSNTS	LPPL PF <mark>KR</mark> GG
3001	G <mark>K</mark> DGSVTGT <mark>K</mark>	RLRCMPAPEE	IVEELPASKK	ORVAPRARGK	SSEPVVIMKR	SL <mark>RT</mark> SAKRIE
3061	PAEELNSNDM	KTNKEEHKLQ	DSVPENKGIS	LRSRRONKTE	AEQOITEVEV	LAE <mark>R</mark> IE IN <mark>R</mark> N
3121	E <mark>KK</mark> PM <mark>K</mark> TS PE	MDIQNPDDGA	RKPIPRDKVT	EN <mark>KR</mark> CL <mark>R</mark> SAR	ONES SOPKVA	EESGO <mark>DK</mark> SA <mark>K</mark>
3181	VLMONO <mark>R</mark> GRG	EAGNSDSMCL	RSRKTKSOPA	ASTLES <mark>K</mark> SVQ	RVTR SVKRCA	EN P <mark>KK</mark> AED NV
3241	CVKKIRTRSH	RDSED				

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 collour) 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 (pdbtm).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).

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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:	KKKARQKLVKNLETFFYALLHSAIM	25	0.5	0.58	0
2:	GSRRRPRAPRMSTAISVLLAQAVFLLLTSQ	29	0.6	0.87	0.6
3:	GSRRRPRAPTAISVLLAQAVFLLLTSQ	27	0.75	0.89	0.2
4:	KRRPRTRAQKMSTAISVLLAQAVFLLLTSQ	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 vielded 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 self-assembly propensity evidence for the 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 macropinocytocis.

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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