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REVIEW ARTICLE

Nano-Inspired Technologies for Peptide Delivery

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Abstract: Nano-inspired technologies offer unique opportunities to treat numerous diseases by using therapeutic peptides. Therapeutic peptides have attractive pharmacological profiles and can be manufactured at relatively low costs. The major advantages of using a nanodelivery approach comprises significantly lower required dosages compared to systemic delivery, and thus reduced toxicity and immunogenicity. The combination of therapeutic peptides with delivery peptides and nanoparticles or small molecule drugs offers systemic treatment approaches, instead of aiming for single biological targets or pathways. This review article discusses exemplary state-of-the-art nanosized delivery systems for therapeutic peptides and antibodies, as well as their biochemical and biophysical foundations and emphasizes still remaining challenges. The competition between using different nanoplatforms, such as liposome-, hydrogel-, polymer-, silica nanosphere-, or nanosponge-based delivery systems is still "on" and no clear frontrunner has emerged to date.

Keywords: Peptide drug, nanodelivery, physiological barriers, toxicity, immunogenicity, therapeutic peptides.

1. INTRODUCTION

1.1. Pros and Cons of Therapeutic Peptides

Therapeutic peptides contain up to 50 amino acids, which corresponds to > 10^{35} possible peptide sequences. Because of their versatility and tunability, and the experience that has been gained during a century of research, peptide therapeutics are considered highly selective, biocompatible and reasonably safe [1-3]. Major problems arise from rapid proteolytic degradation, peptide adsorption (*e.g.* to serum proteins), (bio) distribution, metabolism, and excretion (ADME) [4] and *in-vivo* pharmacological barriers [1]. These effects have severely limited clinical translation, especially when delivery to the brain [5] and/or tumors/metastases [5, 6] has been attempted.

Here, we will address the challenges and opportunities of using nanotechnology-based approaches to improve the pharmacological profile [3] of therapeutic peptides [7, 8]. Historically, peptides have been developed to target G-protein coupled receptors [9] and other extracellular targets, ion channels and enzymes, such as proteases and kinases [10-14]. In these areas, major competition by small-molecule drugs exist, which can be synthesized more efficiently and are more cost-efficient [15, 16]. Furthermore, high-throughput screening methods for small-molecule drugs exist that can identify lead compounds from large libraries [17, 18]. These disadvantages have been relatively recently addressed by developing renewable methods for peptide synthesis [19], and incorporation of non-natural building blocks [20, 21] and the design of peptide/drug hybrids [22-26]. A major advantage of therapeutic peptides in comparison with small molecule drugs is that they are capable of effectively targeting protein: protein interactions (PPIs), especially if these interactions depend on numerous spatially-distinct low affinity interactions, in opposite to PPIs that depend on pockets or concentrated binding foci [1, 10, 27-30]. Further application of peptide therapeutics comprise cell signaling [31], and inhibition of cell function [32].

"According to the *Global Peptide Therapeutics Market & Clinical Trials Insight 2022* report [33], there are more than 100 peptide-based drugs commercially available, and 688 of them are in the clinical pipeline and have variety of different delivery strategies" [1].

1.2. Peptide Toxicity and Immunogenicity

One major limitation of therapeutic peptides is their potential toxicity towards eukaryotic cells, as well as their immunogenicity. The peptide community has experienced the emergence of *in-silico* tools for toxicity and immunogenicity prediction and advanced peptide design, which offer the opportunity to fine-tune peptide properties [34]. Principally, computational resources can be divided into databases and

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in-silico models for toxicity prediction. Among the former, ATDB (Animal Toxin Database) [35, 36], VFDB (Virulence factor database) [37, 38], DBETH (Database of Bacterial Exotoxin for Human) [39, 40], and especially UnitProtKB [41, 42] are widely used for the identification of known toxic or immunogenic peptide sequences. There is a rapid increase of *in-silico* models for peptide toxicity prediction in the literature [34]. The following three *in-silico* models are most noteworthy in the opinion of the authors: BTXpred uses vector machine-based models, which can be combined with hidden Markov models (HMM) and PSI-BLAST, to predict bacterial toxins, as well as to identify them as exo- or endotoxins [43, 44]. NTXpred consists of a SVM model in combination with PSI-BLAST. It performs amino-acid and dipeptide combination-based predictions of neurotoxins [45, 46]. ToxinPred is probably the most user-friendly *in-silico* method for peptide toxicity prediction [47, 48]. It permits the design and toxicity evaluation of multiple single peptide mutations. It is noteworthy that even the best *in-silico* methods are not yet perfect. Therefore, peptide synthesis, followed by experimental determination of peptide toxicity are mandatory. The following methods are used: Lactase dehydrogenase (LDH) Leakage Assay [49], MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) Assay [50], Hemolytic Assay [51], as well as ATP-based Assays [52]. Based on the outcomes of the *in-silico* methods and/or experimental determination of peptide toxicity, the following established synthesis strategies can be used to improve their therapeutic indices: Peptide-mutation aimed at improving their biophysical properties [34], inserting D-amino acids [53-56], synthesis of retro-inverso peptides [57-59], inserting chemically modified (non-natural) amino acids [60], cyclization [53, 61], and end modifications, for instance C-amidation and N-acetylation [62]. With respect to immunomodulation, it should be noted that approx. 30% of licensed pharmaceutical products are biologics that target autoimmune and inflammatory diseases and cancer [63]. Whereas immunogenicity is a "feature" in these applications, it is considered a "bug" in virtually all others. It is anticipated that the synthetic strategies discussed above will be capable of lowering the immunogenicity of therapeutic peptides, without significantly lowering their efficacies. Furthermore, the effective delivery of therapeutic peptides will be the key to suppressing immunogenicity, because this approach will allow a significantly decrease in their dosages.

1.3. Proteolytic Instability

Intracellular peptidases are capable of degrading more than 99% of the peptides that are either released by the proteasome or were able to enter the cell [64] within minutes [3]. The following proteases have been identified as key players in these processes: Tripeptidyl peptidase II (TPPII) [65], Thimet oligopeptidase (TOP) [66, 67], Neurolysin [68], Nardilysin (insulin-degrading enzyme) [69] and Prolyl oligopeptidase [70]. The peptides generated by peptidases are further converted into free amino acids by aminopeptidases, including puromycin-sensitive aminopeptidase, leucine aminopeptidase, cysteinylaminopeptidase, insulin-regulated aminopeptidase, bleomycin hydrolase, aminopeptidase A and aminopeptidase B [71, 72]. In serum, metallo- and Ca^{2+}-dependent proteases are present (*e.g*. metalloproteinases), as well as heparin (*e.g*. thrombin, factor Xa) and plasmin [73] that all exhibit proteolytic activity. They are responsible for the effective proteolytic degradation of peptides in blood circulation. Important interstitial proteases comprise collagenolytic enzymes, which include matrix metalloproteinases, cathepsin K, and neutrophil elastase [74].

To enhance the pharmacological profile of linear therapeutic peptides, the following strategies have been explored:

1). Chemical Modification of Peptides

2). Synthesis of Cyclic Peptides

3). Supramolecular Aggregation of Therapeutic Peptides

4). Development of Nanocarriers for Therapeutic Peptides

1.4. Uptake Mechanisms of Nanoparticles in Cells

The uptake of peptides, proteins, and nanostructures by cells occurs by means of various pathways, depending on their size and, to a lesser degree, on their shape, albeit the discussions about shape are somewhat clouded by persisting problems of reproducibility in nanostructure synthesis [1]. Principally, all of these pathways can be exploited for the delivery of nanoformulations. Before the development of novel transport vectors for drugs, genetic material, nanoparticles or peptide/protein therapeutics, the cellular uptake mechanisms and their typical uptake kinetics should be considered. This is especially important if defensive cells or stem cells will be used as transport vectors to cancers or sites of inflammation and/or bacterial infection [75-77]. The three principal pathways of endocytosis can be distinguished as non-specific uptake, pinocytosis, and phagocytosis [1, 78-85] (Fig. **1**). Non-specific uptake occurs *via* direct penetration, the formation of transmembrane pores, or passive diffusion [81-85]. Pinocytosis (cellular drinking) consists of the uptake of fluid and dissolved (macro)molecules *via* small vesicles (< 0.15 μm in diameter) [78]. Depending on the size of the object that is ingested *via* pinocytosis, macropinocytosis, clathrin- and caveolin-mediated endocytosis, and clathrin- and caveolin-independent endocytosis can be distinguished [79]. Phagocytosis, which proceeds *via* the formation of phagosomes (> 0.25 μm in diameter) [79, 80] is utilized for the assimilation of microorganisms and cell debris and mainly performed by phagocytes (*i.e*., monocytes/ macrophages, neutrophils, dendritic cells). Phagosomes undergo fusion and fission events with components of the endocytotic pathway. Eventually mature phagolysosomes are formed, in which their content is enzymatically degraded. [86]. Macropinocytosis depends on actin formation. Macropinosomes are large intracellular vesicles [87, 88].

Clathrin-mediated endocytosis belongs to the group of receptor-mediated endocytotic processes. It is distinctly faster than phagocytosis, micropinocytosis, and caveolin-dependent endocytosis. In virtually all mammalian cells, clathrin-mediated endocytosis is responsible for nutrient uptake, for instance cholesterol-laden low-density lipoprotein particles that target the low-density lipoprotein receptor and iron-rich transferrin that binds to transferrin receptors [89, 90]. Caveolae are flask-shaped invaginations of the plasma membrane. Caveolin-mediated endocytosis is dependent on cholesterol-rich microdomains (lipid rafts, 40-50nm in di-

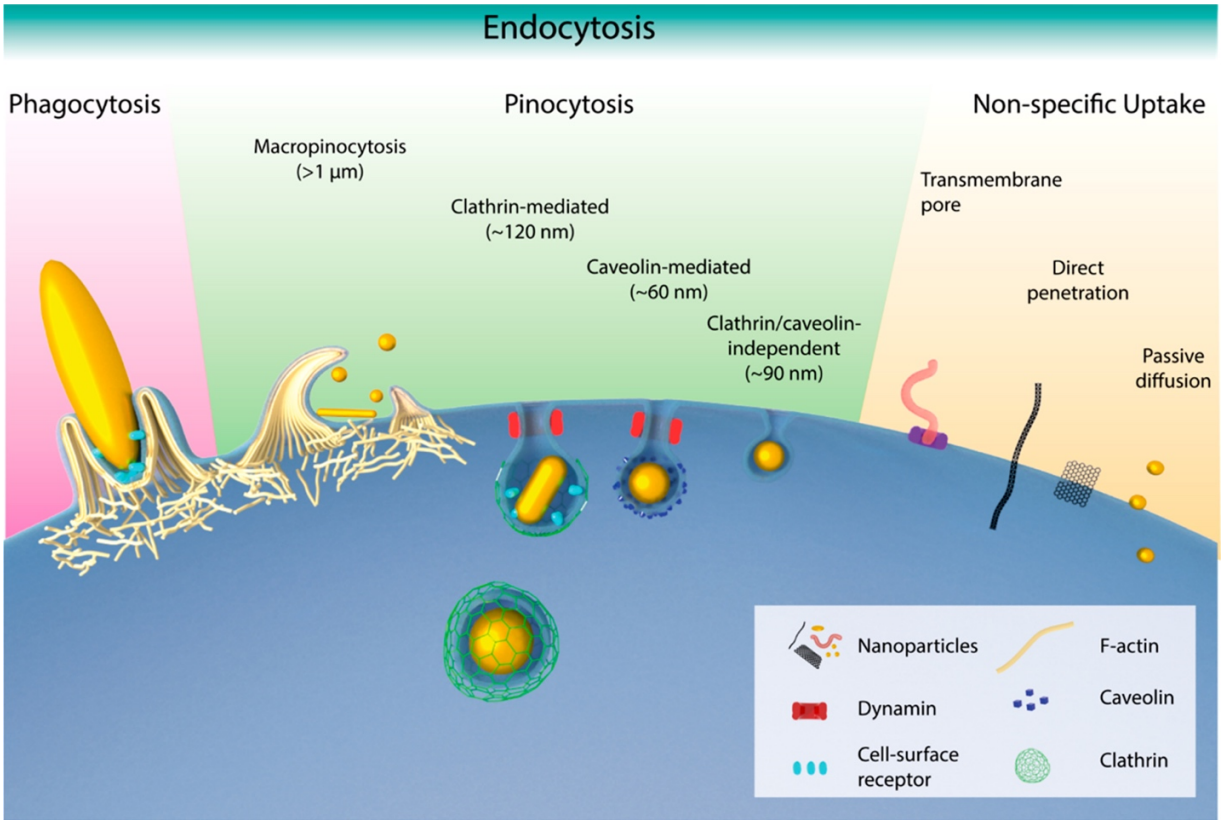

Fig. (1). "Schematic representation of different endocytotic mechanisms. Large (micrometer-sized) particles may be actively incorporated *via* phagocytosis. Areas of high curvature on anisotropic particles, such as large ellipsoids, can contact cells and be more favorably phagocytosed. Smaller particles can be internalized through multiple distinct mechanisms, namely, macropinocytosis (>1 μm), clathrin-mediated endocytosis (~120 nm), clathrin- and caveolae-independent endocytosis, or caveolae-mediated endocytosis (~60 nm). Besides active transport, nanoparticles may also enter the cell passively *via* diffusion or passive uptake by van der Waals or steric interactions through the plasma membrane. This can include piercing of the cell membrane by areas of very high curvature (*e.g.*, carbon nanotubes or graphene edges)" [96]. Reprinted with permission from (Kinnear C, Moore TL, Rodriguez-Lorenzo L, Rothen-Rutishauser B, Petri-Fink A. Form Follows Function: Nanoparticle Shape and Its Implications for Nanomedicine. Chem Rev (Washington, DC, U S). 2017;117(17):11476-521. doi: 10.1021/acs.chemrev.7b00194). Copyright (2017) American Chemical Society. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

ameter) [91, 92]. Caveolin-1 is a dimeric protein that binds cholesterol for lipid homeostasis [93]. Clathrin- and caveolin-independent endocytotic mechanisms are less understood than receptor-mediated uptake processes. The general paradigm is that they too arise from cholesterol-rich microdomains and proceed *via* sorting processes of membrane proteins, glycoproteins, and/or glycolipids [91, 92]. All pinocytotic and phagocytotic processes have in common that the proteins and nanoparticles end up in membrane-bound compartments. Therefore, appropriate "escape strategies" have to be designed to avoid enzymatic degradation of the delivered payload in late endosomes, which are formed by means of fusion between endosomes and lysosomes [3, 94, 95].

1.5. Interactions of Peptides with Cell Membranes

In opposite to cell-penetrating peptides, bilayer-disrupting peptides are therapeutic peptides, which kill cells by means of one of four principal mechanisms of bilayer membrane disruption [81]. Upon reaching the required threshold concentration, the peptides either insert into the bilayer membrane structure and form either barrel stave pores [82], toroidal pores [83], or disordered toroidal pores [84]. For barrel stave and toroidal pores, it is required that the lengths of the peptide sequence after assuming its 3D structure are long enough to span the entire diameter of the bilayer membrane [81]. If that is not possible, disordered toroidal pores can be formed [84]. One important alternative to pore-forming mechanisms is the carpet mechanism [85], in which the therapeutic peptides adsorb parallel to the bilayer. Upon reaching the threshold concentration, micellization of membrane components and entire membrane sections can be observed. These processes can disintegrate bilayer membranes very efficiently [81].

2. CELL PENETRATING PEPTIDES

The discovery of cell penetrating peptides (CPPs) is one of the major breakthroughs in transportation of peptides, proteins and nanostructures through cell membrane. Transport of these large molecules, aggregates and clusters was challenging due to their size and biophysical properties [97]. In 1988, Frankel and Pabo discovered the rapid uptake of a purified *trans*-activator protein (TAT) from HIV virus type 1 by HL3T1 cells [98]. Its rapid uptake was later to be discovered to occur through endocytosis [99]. In 1997, Vives *et al*.

discovered that the domain responsible for cellular uptake was a region that consisted of 13 amino acids (GRKKRRQRRRPPQ) [100]. Park *et al*. then successfully shortened the TAT peptide to the 9-mer RKKRRQRRR in 2002 [101, 102]. To date, CPPs are relatively short peptide sequences with 5-30 amino acids that can go through tissue and cell membranes, either by direct penetration (energy independent) or endocytosis (energy dependent) [99, 102] (Table **1**). CPPs are unique, since they can not only penetrate cells, but they can also transport a variety of cargos, including: proteins, peptides, small drugs, nanoparticles, as well as genetic information like DNA and siRNA [102]. Despite being a breakthrough due to their efficiency to penetrate cells and transport payloads, the first generation of CPPs did not show a significant specificity for any targeted tissue, which is a major drawback for their intended application of delivering cargo to cancer/metastases or pathogens [2, 102]. Therefore, in order to enhance cellular uptake to specific sites, scientists have developed stimuli-response CPPs, which stimuli can be responsive to pH or stress variation as well as enzymic activity [103]. For example, a CPP was designed and synthesized to form an α-helical structure that became pH responsive by substituting all lysine with histidine [104]. This study demonstrated that at physiological pH, the peptide remained at a neutral charge, and when exposed to acidic conditions, its net charge became positive, which then activated the ability to penetrate cells [103].

In general, cationic, hydrophobic, and amphipathic CPPs can be distinguished.

2.1. Chemical Stabilization Strategies for Therapeutic Peptides

2.1.1. Chemical Modification

Numerous chemical stabilization strategies of therapeutic peptides are discussed in the literature. All of these modifications are designed to decrease their vulnerability to proteolytic cleavage and, therefore, to enhance their circulation lifetimes. Among the simplest chemical modifications are N-terminal acetylation or glycosylation, and C-terminal amidation [62]. Other strategies comprise N-methylation [129], integration of D-amino acids [53-56], β-amino acids [130], β– and γ–amino acids [131], and incorporation of non-native amino acids and pseudo-peptide bonds [60]. Complete D-amino acid variants, synthesized as "retro-inverso" peptides [57-59], exhibit significantly enhanced *in-vivo* stability (>10 times higher circulation lifetimes) [132, 133]. However, decreased delivery activity of retro-inverso peptides has been observed *in-vivo* [134].

It has been established that the presence of sulfated or phosphorylated tyrosine residues and linkage of N-terminal glutamic acid *via* the γ-carboxy group decreases proteolytic activity as well. [135]. A widely established strategy to enhance peptide stability is the attachment of polyethylene glycol (PEG) [136]. This chemical modification increases peptide hydrophilicity and reduces immunogenicity [137]. This concept was successfully demonstrated by investigating HIV-1 fusion inhibitors [138] and BH3 peptide [139, 140], which showed significant improvements in half-lives, compared to the chemically not modified peptides [138-140]. The major drawbacks of chemical modifications are that the increase in half-lives seldom exceeds a factor of five, and that chemical modification can either impair or modify peptide function [1, 3].

2.1.2. Cyclic Peptides

Cyclisation is a viable strategy to suppress exoproteolytic activity by eliminating terminal peptide bonds and to decrease endoproteolytic activity by creating rigidity in the formed macrocycle [53, 61]. The most effective targeting peptides to date are the group of iRGD-derivatives, which have been pioneered by Ruoslahti [91, 141-149]. The development of the cyclic iRGD follows the discovery of the tripeptide sequence Arg-Gly-Asp (RGD) [150] (Table **2**) that is the most acclaimed recognition motif for αν integrins. The latter are members of a family of 24 receptors that facilitate cellular adhesion to and migration on extracellular matrix proteins [151]. $\alpha_v\beta_3$ is one of the αν integrins that RGD is able to target. It is highly expressed on endothelial cells of tumor neovasculature, as well as on the surface of some tumor cells [151]. However, RGD is also able to target $\alpha_v\beta_1$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, and $\alpha_{IIb}\beta_3$, which is the cause for limited targeting efficacy [151, 152]. Nevertheless, RGD-mediated targeting has been extensively explored for cancer imaging and drug delivery [153, 154]. Cyclic RGD-derivatives offer the advantage of enhanced stability and binding strength to αν integrins [153, 155]. In 2009, Ruoslathi *et al*. published the cyclic peptide iRGD (i=internalizing) CRGDK/RGPD/EC), which is capable of effective cell/tissue penetration in addition to tumor-targeting by means of RGD [156] (Figs. **2** and **3**). The family of cyclic iRGD derivatives has proven superior to other targeting peptides in tumor-targeted delivery of small molecules, antibodies, and nanoparticles [143-147, 156, 157]. The enhanced performance of iRGD can be explained by demonstrating that it follows consecutive steps [156]: In the first step, the RGD motif on the N-terminal region of iRGD recognizes $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins. iRGD is then proteolytically cleaved to reveal the cryptic C-end Rule (CendR) motif (R/KXXR/K, X=any amino acid) [156]. Thus, the N-terminal half fragment of iRGD (CRGDK/R) is enabled to bind to neuropilin-1 (NRP-1), which facilitates cellular uptake, vascular leakage, and deep penetration into extravascular tumor tissue [141]. The family of neuropilines (NRPs) are trans-membrane receptors required for axon guidance and vascular development. They feature a carboxy-terminal, which recognize growth factors and other hormones through a carboxy (C)-terminal (CendR binding motif). Peptides featuring this motif trigger receptor-mediated endocytosis. Interestingly, CendR-mediated endocytosis resembles macropinacytosis. It has been labeled a "bulky transport pathway". Cho *et al*. have demonstrated in 2019 that both, the N- and C-termini of iRGDC can be tethered to either a FRET-pair to enable imaging or the combination of a fluorescent dye (cyanine 5.5) and a cisplatin prodrug, thus creating a theranostic reagent based on iRGD [157].

2.2. Delivery to the Central Nervous System

The blood-brain-barrier (BBB) [2, 158] is an essential biological semipermeable membrane that shields the brain and central nervous system (CNS) from peripheral circulating blood [159]. The BBB is formed by complex interaction

Table 1. Cell-Penetrating Peptides (CPPs), ordered by biophysical properties [2, 102, 105].

Classification	Peptide	Sequence	References
Cationic	TAT (HIV-1 TAT protein, TAT_{48-60})	GRKKRRQRRRPPQ	[100]
	TAT (HIV-1 TAT protein, TAT_{49-57})	RKKRRQRRR	[101]
	Penetratin $pAntp_{43-58}$	RQIKIWFQNRRMKWKK	[106, 107]
	Polyarginines	R_n	[108]
	Polylysines	K_n	[109]
	DPV1047	VKRGLKLRHVRPRVTRMDV	[110]
	[D]-K6L9	LKlLKkLlkKLLkLL	[54, 85]
	NLS	CGYGPKKKRKVGG	[111]
	cyclic $[W(RW)_4]$	[WRWRWRWRW]	[112]
	NrTP5	ykqchkkGGkkGsG	[113]
	hPP3	KPKRKRRKKKGHGWSR	[114]
Amphiphatic	Transportan	GWTLNSAGYLLG GWTLNSAGYLLGKINLKALAALAKKIL	[115, 116]
	MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	[117]
	Pep-1	KETWWETWWTEWSQPKKKRKV	[117]
	VP22	NAATATRGRSAASRPTQR	[118]
	MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	[117]
	pVEC	LLIILRRRIRKQAHAHSK	[119]
	BPrPp (1-26)	MVKSKIGSWILVLFVAMWSDVGLCKKRP	[120]
	ARF (1-22)	MVRRFLVTLRIRRACGPPRVRV	[121]
	VT5	$DPKGDPPKGV(TV)_5GKGDPKPD$	[122]
	MAP	KLALKLALKALKAALKLA	[123]
	p28	LSTAADMQGVVTDGMASGLDKDYLKPDD	[123]
	Bac 7 (Bac_{1-24})	RRIRPRPPRLPRPRPRPLPFPRPG	[124]
Hydrophobic	C105Y	CSIPPEVKFNKPFVYLI	[125]
	PFVYLI	PFVYLI	[126]
	Pep-7	SDLWEMMMVSLACQY	[127]
	VP22	DAATATRGRSAASRPTERPRAPARSASRPRRVD	[128]

Table 2. The iRGD Family.

Year	Peptide	Sequence	References
2009	iRGD	CRGDK/RGPD/EC	[156]
2013	Cys-iRGDC (iRGDC)	C X'CRGDK/RGPD/EC (X' = 6-aminohexanoic acid)	[145, 157]
2013	Cys-X-iRGD (iRGDC)	CX'GGSGGSGGCRGDK/RGPD/EC (X' = 6-aminohexanoic acid)	[145]

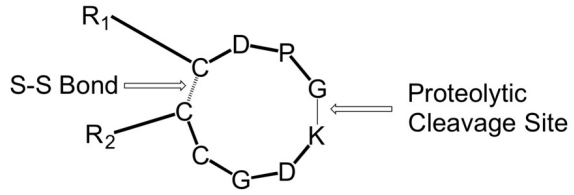

Fig. (2). General Structure of the iRGD Family: iRGD contains a proteolytic cleavage site and a disulfide bond, which undergoes reductive cleavage [156]. On both, the N- and C-termini, functional tethers and/or diagnostic or therapeutic payloads can be attached [157].

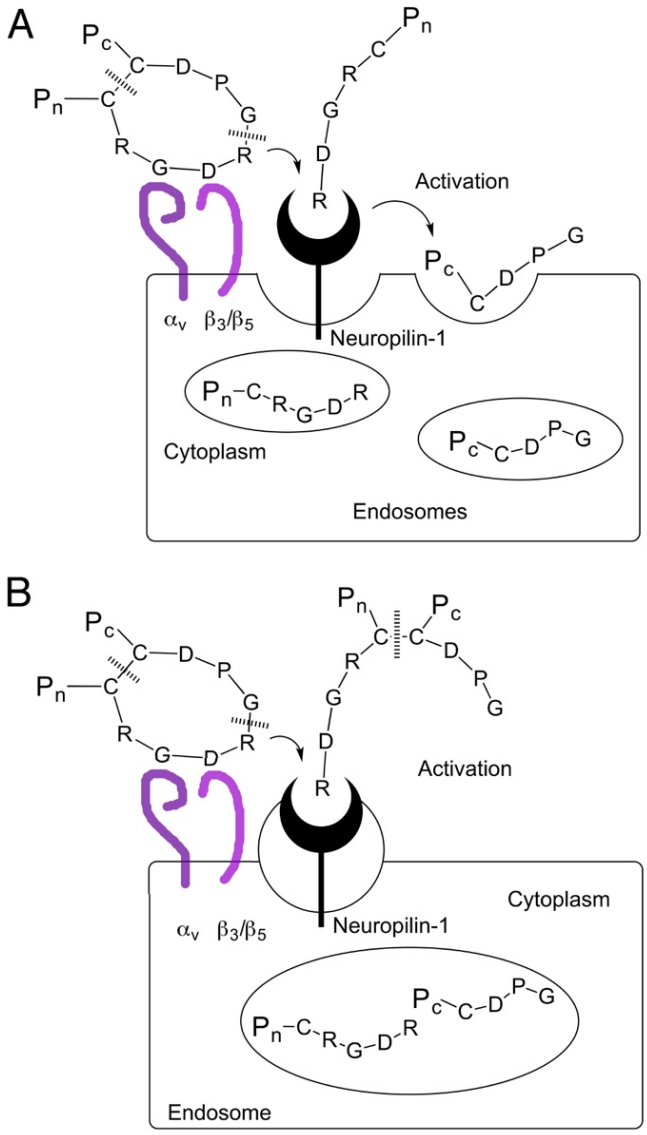

Fig. (3). Proposed mechanisms for iRGD uptake: (**A**) extracellular proteotic and reductive cleavage, followed by endosomal uptake; (**B**) extracellular proteotic cleavage, followed by neuropilin-1-triggered endosomal uptake and intracellular reductive cleavage. P_c: payload at the C-terminal end; P_n: payload at the n-terminal end [157]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

between vascular endothelium, pericytes, and astrocytes. It is characterized by tight junctions and the absence of fenestrations, as well as high proteolytic activity and the existence of efflux pumps. Consequently, the BBB effectively restricts transport for numerous solutes [159]. Naturally, this also means that numerous therapeutic compounds cannot cross the BBB into the CNS [160]. Passive transport of gases, water, and other small molecules (*e.g.* ethanol) is quite effective. Passive transport of larger molecules is governed by molecular size, polarity (logP) and charge. A general rule (with numerous exceptions) is that molecules that are able to cross the BBB by means of passive diffusion should have a molecular weight of 400-500, log P = 1-2, and basic pH [161-163]. However, the shape of molecules and especially peptides can be quite a decisive factor [164]. CPPs hold enormous promise for delivery across the BBB, in spite of negative experiences in the past [2, 165]. Schwarze *et al.* demonstrated in 1999 that Tat is able to deliver conjugated β-galactosidase into the mouse brain [166]. This finding is regarded as the beginning of designing Tat-derivatives and other peptides for enhanced BBB crossing [167-169]. As Table **3** indicates, numerous peptides have been inspired by peptide shuttles that were designed by evolutionary processes to deliver essential nutrients across the BBB. For instance, transport peptides capable of triggering receptor-mediated transcytosis (RMT) involving nicotine acetylcholine (nAChRs) [170], transferrin (TfR) [171], low-density lipoprotein (LDLR) or low-density lipoprotein receptor-related protein-1 (LRP-1) [170, 172] were designed to deliver payloads to the CNS. The discovery that endogeneous ligands are transported through the BBB [173] sparked an intensive search for suitable peptides, which are also summarized in Table **3**. The 16-residue peptide CDX, derived from the snake neurotoxin candoxin, binds to nicotine acetylcholine receptors (nAChRs), which facilitates transcytosis [174]. It is noteworthy that the retro-inverso isomer (^{re}CDX) (= the inverted sequence of D-amino acids) displayed superior transcytosis efficacy compared to ^{L}CDX when crossing to BBB of glioblastoma-bearing nude mice [174], because of its significantly higher stability against proteolytic degradation. However, it should be noted that glioblastomas usually cause at least a partial breakdown of the BBB, especially in later stages [175]. The transferrin receptor TfR internalizes can be targeted by peptides B6, CRT, THR, and T7, which have been discovered through page displays [176-179]. All four peptides mimic the transferrin-iron complex, which is naturally shuttled by TfR through the BBB.

2.3. Antibody-Mediated Uptake of Therapeutic Peptides

Antibodies hold considerable promise for targeting "soft targets", which do not exhibit structures that are druggable using small molecule drugs [189, 190]. They can be generated by means of the well-established hybridoma technology or phage displays [191, 192]. Although the general technology is well-established, the industrial production of antibodies with consistent quality standards can be challenging at times [193]. Whereas targeting epitopes at the surface of cells is straightforward, targeting cytosolic targets requires uptake [189]. These antibodies can be taken up by phagocytotic cells, sometimes causing off-target effects [194]. This problem can be addressed by utilizing smaller antibody fragments devoid of Fc regions such as antigen binding fragments (Fab), single chain variable fragment (S_cF_v), and nanobodies [189]. As discussed above, smaller antibody derivatives will be cleared from circulation much more rapidly

Table 3. Peptides Capable of Transcytosis Through the Blood-Brain-Barrier.

Proposed Transport(s)	Peptide BBB Shuttles	Sequence	Refs.
nAChRs	RVG29	YTIWMPENPRPGTPCDIFTNSRGKRASNG	[180]
-	^{L}CDX	FKESWREARGTRIERG	[174]
-	^{re}CDX	GreirtGraerwsekf	[153]
LRP/LDLR	Angiopep-2	TFFYGGSRGKRNNFKTEEY	[181]
-	ApoB (3371–3409)	SSVIDALQYKLEGTTRLTRKRGLKLATALSLSNKFVEGS	[182]
-	ApoE $(159–167)_2$	$(LRKLRKRLL)_2$	[183]
TfR	B6	CGHKAKGPRK	[177]
-	T7	HAIYPRH	[176]
-	THR	THRPPMWSPVWP	[179]
-	^{re}THR	pwvpswmpprht	[184]
Leptin Receptor	Leptin 30	YQQILTSMPSRNVIQISND-LENLRDLLHVL	[185]
GSH transporter	GSH	γ-l-glutamyl-CG	[184]
GM1	G23	HLNILSTLWKYRC	[185]
AMT	Tat(47-57)	YGRKKRQRRR	[186]
-	SynB1	RGGRLSYSRRRFSTSTGR	[187]
Active transport	PepNeg	SGTQEEY	[188]

Abbreviations: AMT, adsorptive-mediated transport; GM1, monosialotetrahexosylganglioside; GSH, glutathione; KCa channel, calcium-activated potassium channel; LDLR, low-density lipoprotein receptor; LRP-1, low-density lipoprotein receptor-related protein-1; nAChRs, nicotine acetyl-choline receptors; TfR, transferrin receptor.

than native antibodies [3, 189]. It should be mentioned that antibody-targeting of cellular surface receptors often leads to receptor-mediated endocytosis [195]. However, this is not an option for an antibody with a target in the cytosol, mitochondria, endoplasmic reticulum, or nucleus [189]. Once cytosolic antibodies are taken up *via* endocytosis, the endosomes are fused with lysosomes, which facilitate cathepsin-mediated degradation of protein content. Therefore, antibodies/ antibody-fragments have to escape from the endosomes to remain active in the cytoplasm [196]. There are several solutions to this problem (Fig. **4**). The antibody (fragment) can either be microinjected [197, 198], or delivered *via* electroporation [199, 200], which is only feasible *in-vitro*, or attached to a delivery vehicle or a peptide sequence allowing rapid uptake by the cell and/or endosomal escape (Table **1**) [189].

3. NANOSCOPIC DELIVERY SYSTEMS FOR ANTIBODIES, ANTIBODY FRAGMENTS, AND PEPTIDES USING MESOPOROUS SILICA NANOPARTICLES

3.1. Delivery of Antibodies and Antibody Fragments

This decade has experienced the rise of mesoporous silica nanoparticles (MSN) as drug delivery agents [201, 202]. MSN possess the following intrinsic advantages with respect to drug delivery: They are fully biocompatible, possess excellent surface functionalization capabilities, and pore volume tenability [201]. In addition, the inherent rigidity of the material can protect encapsulated antibodies against pH-changes and enzymatic degradation [203]. For instance, IgG antibodies (immune-globulins) can be absorbed in MSN pores [204]. This is possible, because the pores of MSN, which are synthesized *via* micelle-templated methods, can be tuned from 2-50 nm [201]. A typical IgG is 13.7 nm in length and 8.4 nm in height [205]. MSNs are also used for the purpose of delivering therapeutic peptide sequences, which requires gatekeepers for triggered drug release [206-209]. Furthermore, circulation lifetimes are significantly increased, because MSN/antibody aggregates are larger than antibodies/fragments alone. Typically, MSN designed for the adsorption of antibodies, *e.g*. Anti-phospho-Akt [204], are about 20 nm in diameter [207]. Uptake *via* energy-dependent endocytotic pathways, such as through clathrin pits and actin filaments is observed. The third class of MSN are hollow dendritic mesoporous silica nanospheres featuring a singular hole per particle of 25-50nm in diameter, which can be filled either with peptides or proteins [210, 211].

3.2. Delivery of Therapeutic Peptide Sequences

As shown in Fig. (**5**), the design of mesoporous silica nanoparticles (MSN) can be adapted to specifications for the purpose of delivering therapeutic peptides [212]. The templated synthesis of MSNs by means of condensation of siloxane-precursors permits the adaptation of MSN sizes and pore sizes to the chosen cellular uptake mode [96]. Furthermore, MSN can be synthesized around core materials, for instance magnetic iron oxide for the purpose of magneto-

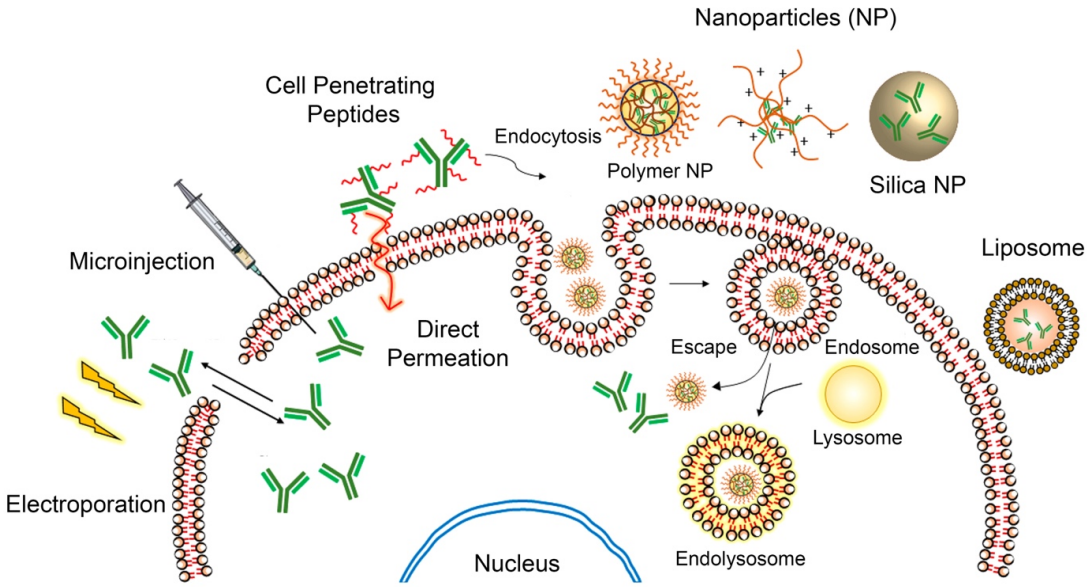

Fig. (4). Mechanism of cellular entry by peptides, antibodies and nanoparticles. Adapted with permission from (Singh K, Ejaz W, Dutta K, Thayumanavan S. Antibody Delivery for Intracellular Targets: Emergent Therapeutic Potential. Bioconjugate Chem. 2019, doi: 10.1021/acs.bioconjchem.9b00025.). Copyright (2019) American Chemical Society [189]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

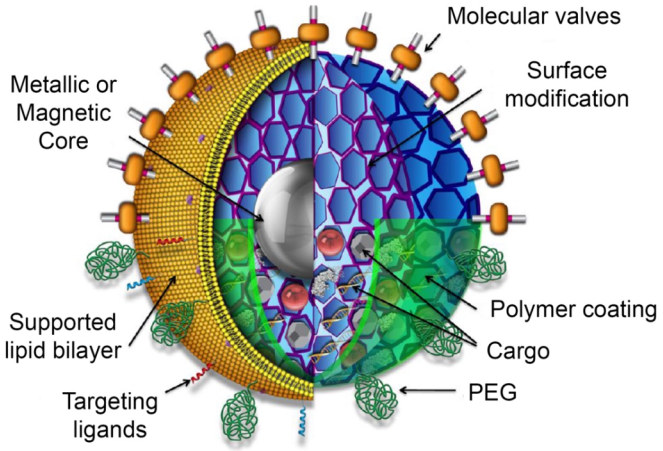

Fig. (5). "Schematic representation of a multifunctional mesoporous silica nanoparticle showing possible core/shell design, surface modifications, and multiple types of cargos." Adapted with permission from (Tarn D, Ashley CE, Xue M, Carnes EC, Zink JI, Brinker CJ. Mesoporous Silica Nanoparticle Nanocarriers: Biofunctionality and Biocompatibility. Accounts of Chemical Research. 2013;46(3):792-801. doi: 10.1021/ar3000986.) Copyright (2013) American Chemical Society [212]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

transfection and MRI [212-214]. The absorption of therapeutic peptides within the channels of the MSN (after template/surfactant exchange) follows a concentration gradient. It can be aided by optimizing the charge of the exterior and/or interior of the MSN by reaction with APTES ((3 Aminopropyl) triethoxysilane and other aminosilanes) [214, 215]. "Gatekeepers" are necessary to prevent the MSN cargo from leaching out during transport [206-209]. Molecular valves and environmentally-responsive polymer coatings are among the most popular gatekeepers [212]. All gatekeepers have in common that they permit the release of the payload once they have been activated. Furthermore, stealth ligands can be attached, which decelerate the recognition and subsequent clearance of the MSN by the reticuloendothelial system, as well as targeting ligands (peptides or antibody (fragments), see above) [212].

The endosomal protease cathepsin B, which is overexpressed in numerous solid tumors [216, 217], is able to cleave the tetrapeptide GF-LG [218]. This cleavage motif can be used in conjunction with a biocompatible polymer (Poly-N-isopropyl-acrylamide (PNIPAM) or PEG diacrylate (PEGDA)) to construct a gatekeeper that can be enzymatically activated during endocytosis [219]. This concept was successfully demonstrated in HeLa cells and 3T3-J2 fibroblasts through releasing doxorubicin and subsequent cell death [219]. Torre *et al*. utilized T22 peptide to target C-X-C chemokine receptor type 4 (CXCR-4) expressing lymphoma

cells to deliver MSNs into the cytosol *via* receptor-mediated endocytosis [220]. The targeting ability of T22 was significantly decreased in the presence of AMD3100 (antagonist of CXCR-4), demonstrating that T22 competes for CXCR-4 with AND3100 [220]. Again, these examples should be regarded as proof-of-principle for very efficient drug-delivery designs. The challenge that remains is to optimize these systems for targeting diseases (especially cancer) in human patients.

3.3. Liposomes

Liposomes are very simple models of cells. They are spherical and feature phospholipid bilayers, which can be either single or multi-layered, and an aqueous buffer filled core [221, 222]. Although they are biocompatible and biodegradable, classic liposomes often do not permit the release of antibodies, therapeutic peptides, or drugs with sufficient spatio-temporal control [223]. Therefore, during the last four decades [223, 224], efforts have been made to liposomes triggerable by (pH changes in tissue [225], redox mediators (*e.g*. ROS in cancers) [226], light to permit photo-triggerable release [227], and temperature (*e.g*. hyperthermia) [228]. Furthermore, PEGylation has significantly prolonged the circulation-half-life of liposomes [229]. Although PEGylated liposomes can remain in circulation significantly longer [230], the efficacy of drug delivery is still hampered by the absence of targeting mechanisms and very varying extravasation efficacy in human patients, in comparison to mouse models [231, 232]. Therefore, the next logical step is to attach ligands, peptides, and antibody (fragments) for specific targeting [229]. However, this "active targeting approach" is either dependent on the availability of targets in blood, at the cell walls of blood vessels, or after extravasation. Unfortunately, tumor heterogeneity is the major roadblock to extravasation and consequent drug delivery [1]. Several nanotherapeutics making use of liposomal formulations have reached the market (Doxil/Caelix, Johnson & Johnson; AmBisome, Gilead; Myocet, Cephalon) [233]. In order to overcome endosomal entrapment and enzymatic degradation, several (model) liposomal systems have been developed: CD44 is a transmembrane glycoprotein (P-glycoprotein) that facilitates receptor-mediated endosomal uptake [234]. Utilizing anti-CD44 antibody decorated liposomes composed of DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), DOPE (2-dioleoyl-sn-glycero-3-phosphoethanolamine), cholesterol, and antibody-labeled 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[succinimidyl (poly-ethylene glycol)-3400] (DSPE-PEG_{3400}-NHS), bearing an anti-interleukin 6 receptor (IL6R) antibody as payload, Guo *et al*. were able to inhibit IL6R-Stat3 signaling in tumor bearing mice [235].

A multifunctional nanocarrier system capable of controlling intercellular trafficking was developed by Yamada *et al*. [124]: The cell-membrane penetrating peptide stearyl-octaarginine (R8) (Table **1**) was used in conjunction with the cholestenyl ester-labeled fusogenic peptide GALA (WEAALAEALAEALAEHLAEALAEALEALAA). GALA adopts a random coil structure at neutral pH, but changes to an amphipatic α-helical structure at lower pH, which is typical for endosomes [236]. Based on this pH-induced change of structure, GALA is a very efficient disruptor of endosomal membranes, leading to fast endosomal escape [236]. Both peptide sequences were anchored using hydrophobic labels in the liposomes' bilayer, which was composed of the surfactant DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) and cholesterol hemisuccinate [237]. Whereas liposome-mediated delivery of protein and peptide payload *via* endocytosis and endosomal escape usually requires 4-24h incubation time, this nanoplatform is capable of completing both steps to 99% of cells within 30-120 min! An alternative strategy to promote fast endosomal escape is to utilize a photosensitizer to trigger ROS (reactive oxygen species)-mediated degradation of the endosomal membrane, thus facilitating endosomal escape [238]. Interestingly, payload delivery by means of membrane fusion, thus avoiding endocytosis, was achieved by a liposomes comprised of DSPE-4A (attached with R4 and DSPE-Hy-PEG2k (attached with benzaldehyde) [239]. Basel *et al*. have demonstrated an alternative drug delivery concept by designing caged hypertonic liposomes that can be activated *via* proteolytic cleavage of the consensus sequence SGRSA, which was incorporated into a "cage" of polyacrylic acid chains [240]. Once the "bar" of this "cage" is cleaved by the protease, the hypertonic liposome releases its payload immediately. The challenge of this approach is the timing of the proteolytic attack. The authors suggest targeting membrane-bound proteases that are located at the surface of cancer cells (*e.g*. MMP9 on CD44 [241] and MMP14 [242]) to enable drug release in the immediate vicinity of tumors [240].

3.4. Limitations of Liposomal Delivery Systems

Although liposomal delivery systems are widely used and a great commercial success [223, 233], they are suffering from intrinsic drawbacks: Their encapsulation efficiencies are very low, especially for hydrophilic payloads. Liposomes are not long-term stable and, therefore, cannot be stored for very long. Liposomes can be destabilized while in circulation by the interaction with serum proteins, which – in addition – promote opsonization. As this is true for virtually all nanostructures, corona formation decreases targeting efficacy by "burying" the targeting antibody (fragments) or peptides under layers of adsorbed proteins. Furthermore, even stealth-liposomes are recognized by the reticuloendothelial system and cleared [243, 244].

3.5. Supramolecular Peptide Nanostructures/Hydrogels

Technically, supramolecular peptide nanostructures for biomedical applications are hydrogels, or at least closely related with this vast group of materials [245]. Hydrogels can form networks, which are at least partially hydrated. These networks can be formed through covalent of or non-covalent interactions. The latter comprise ionic interactions, hydrogen bonds, or hydrophobic interactions [246]. The resulting hydrogels can be classified as amorphous, semi-crystalline, or crystalline [245] individual components from which hydrogels are formed can be either nonionic or ionic (anionic, cationic), ampholytic (containing both acidic and basic functional groups), or zwitterionic (polybetaines, containing both, positive and negative charges, but not necessarily the same number of them). With respect to this review,

hydrogels formed from peptides belong to the group of hydrogen-forming natural peptides, even if the peptides discussed here are designer peptides. Synthetic polymers are usually formed by means of chemical polymerization [1]. Numerous synthetic hydrogel systems have been designed for drug delivery, which would exceed the capacity of this review [1]. Here, we will focus on peptides that were designed for self-assembly as a strategy to slow down proteolytic degradation and thus significantly enhance circulation time [3].

3.6. Supramolecular Nanofibers, Crossing the Blood-Brain-Barrier

As discussed above, one of the most challenging tasks of drug delivery is the requirement to cross the blood brain barrier [247]. Multiple nanocarriers like liposomes and polymers have been synthesized for delivery to the brain, but no vector has been uniquely successful to date [159]. However, the use of peptides for drug delivery across the BBB hold promise. Mazza *et al*. have demonstrated that transport into the brain using peptide nanofibers can be achieved [248] by designing an amphiphilic peptide-derivative featuring a highly positive charged and hydrophobically labeled terminus (palmitoyl-GGGAAAKRK) [248]. This peptide-derivative self-assembled into peptide nanofibers (PNFs). This self-assembling process generated flexible and elongated nanofibers, which were then incubated with 0.05% trypsin-EDTA to determine possible carboxypeptidase-mediated degradation sites. Images were captured using TEM at different time points. These experiments demonstrated complete proteolytic degradation after 14 days [248]. PNFs were then labeled with Nile Red (hydrophobic fluorescent dye), and then incubated for 24 hours with primary neurons isolated from rat brains. Here, fluorescent microscopy demonstrated the presence of PNFs in the cytoplasm of these cells. Finally, a pilot *in vivo* study was designed to demonstrate localization and possible degradation of PNFs in rats' brains. Rats were intracranial injected with fluorescently labeled PNFs (VivoTag 680 XL), and the occurring fluorescent signal was measured for 15 days using an IVIS fluorescence imaging camera. A strong signal was detected for up to seven days, which almost disappeared after 15 days, but remained close to the site of injection. More mechanistic studies are required to further verify the underlying paradigm, according to which the peptide nanofiber that was not only able to reach the brain, but also was degraded overtime by plasma membranes. This would mean that potential cellular toxicity is strongly reduced by limiting the accumulation of this nanocarrier in the brain [248].

3.7. Cancer Chemotherapy

Chemotherapy is the most used treatment against cancer [249]. However, it is far from optimal due to the lack of tissue specificity, thus causing severe side effects, as well as generally low concentrations of drugs released at the tumor site, thus abetting the development of drug resistance. One of the most interesting applications of self-assembled peptides is an injectable hydrogel formulation, which can place the chemotherapeutic agents next to the target tissues for a higher local concentration release over time [250, 251] Previously, a peptide with alternating ionic hydrophilic and hydrophobic amino acids was reported to form stable β-sheet structures, which was called KLD12 (KLDLKLDLKLDL) [251]. Later, KLD was used to design a protease-sensitive hydrogel with a cleavable region, which facilitated a drug release mediated by trypsin [251]. Yishay-Safranchik *et al*. developed injectable *in situ*-forming hydrogels designed from self-assembled KLD motifs to control the release of doxorubicin (DOX) or Smac-derived pro-apoptotic peptide (SDPP (AVPIAQ)) for cancer treatment. These motifs were designed to be separated into two β-sheet peptides by the following spacers: 3- or 4- glycine (G) spacers or 4- glycine and a phenylalanine (F) spacer. These spacer modifications were intended to increase the gel formation rate, and the use of phenylalanine was intended to improve drug loading and stability by increasing the hydrophobicity of the hydrogel. Results demonstrated that the addition of G spacers decreased the time for gel formation to 3 and 4 minutes after adding PBS, while addition of G and F increased the gel formation rate to only 2 minutes after the addition of PBS. Cell penetration was confirmed on SK-OV-3 (ovarian cancer) cells after they were incubated for 24 hours with hydrogels loaded with SDPP labeled with a fluorescent dye. After 24 hours of incubation 80-90% of cells were fluorescent. Cytotoxicity of DOX being released from KLD-based hydrogels was then analyzed through MTT assays. Results demonstrated that within 24 hours of incubation, 50% cells treated with free DOX were alive, while 60% remained alive for DOX-loaded KLD hydrogels, which remained similar even after 48 and 72 hours. Results demonstrate that DOX release from hydrogels is controlled, since cytotoxic activity was maintained.

Toft *et al*. have designed peptide amphiphiles (PA), composed of hydrophobic, hydrogen-bonding, and hydrophilic domains that self-assemble to form cylindrical nanofibers (Fig. **6**). This supramolecular system has shown promising results to be used as a potential cancer therapeutic [252]. A cationic peptide sequence $(KLAKLAK)_2$ was designed to interact with lipid membranes as well as lyse either plasma or mitochondrial membranes [252]. By conjugation of KLAK to lauric acid $[C_{16}A_4G_3(KLAKLAK)_2]$ cylindrical nanostructures capable of disrupting cell membranes, were assembled. This is very interesting because in this case, KLAK peptide is both the delivery vector and the therapeutic peptide sequence [17]. The drawback of this is that peptides are easily degraded, as discussed above [250]. To protect peptides from proteolysis this therapeutic-delivery vehicle was modified with pegylated peptide (PEG), to create a protective corona [252]. After KLAK was co-assembled to PEG, enzymatic degradation was measured using the protease trypsin. Results demonstrated that percentage of intact KLAK peptide increased as the concentration of PEG peptide increased. Then, cytotoxicity was compared for KLAK alone and KLAK/PEG peptides using MTT assays (reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) by metabolic NAD(P)H-dependent cellular oxidoreductase [25]. MTT assays revealed similar toxicity of both peptides on three cell lines of human breast cancer. Lastly, using mouse breast cancer models, KLAK and KLAK/PEG were administered *via* intraperitoneal injections. Cell proliferation was measured using immunohistological staining for bromodeoxyuridine,

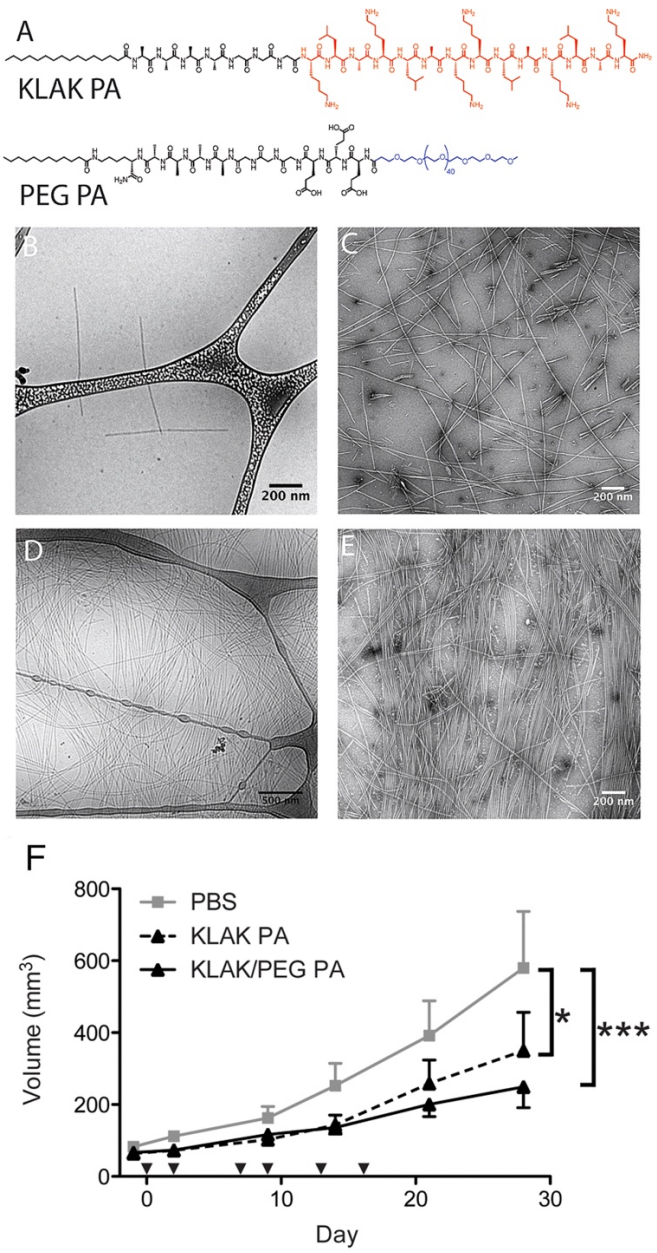

Fig. (6). (**A**) Chemical structure of "KLAK PA" with the sequence palmitoyl-A4G3(KLAKLAK)2 and "PEG PA" with the sequence PEG2000-E3G3A4K(C12). (**B**) Cryo-TEM of KLAK PA alone." (**C**) Cryo-TEM of KLAK (**D**) Cryo-TEM of KLAK with PEG. (**E**) Cryo-TEM of KLAK PA with PEG PA. "(**F**) The growth of MDA-MB-231 human breast cancer orthotopic tumors is inhibited by intraperitoneal treatment (inverted arrows) of KLAK PA nanostructures. Both the KLAK- and KLAK/PEG PA-treated tumors were statistically smaller, as determined by two-way ANOVA." Adapted with permission from (Toft DJ, Moyer TJ, Standley SM, Ruff Y, Ugolkov A, Stupp SI, Cryns VL. Coassembled Cytotoxic and Pegylated Peptide Amphiphiles Form Filamentous Nanostructures with Potent Antitumor Activity in Models of Breast Cancer. ACS Nano. 2012;6(9):7956-65. doi: 10.1021/nn302503s.) Copyright (2012) American Chemical Society [252]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

which showed a lower proliferation for tumors treated with KLAK/PEG [252]. These experiments can be regarded as proof-of-principle that biodegradable nanocarrier can be designed using peptides as key ingredients.

3.8. Peptide Nanosponges

The Bossmann Group has designed peptide nanosponges for drug delivery across physiological barriers and targeting of defensive cells in peripheral blood [25, 253, 254]. They consist of poly-K or poly-R segments (n=5, 10, 15, or 20), a consensus sequence for a protease (*e.g.* DEVDGC for the executioner caspases 3, 6, and 7), as well as a trigonal linker capable of reacting with the linear peptides *via* Michael addition. Peptide nanosponges based on poly-K or poly-R units form spontaneously in aqueous buffers. Formulations can be also amended by mixing various amounts of poly-D nanosponge with either poly-K-, or poly-R-based sponges [254]. Due to charge attraction between the poly-D- and the poly-K-segments, nanosponge-like structures are formed featuring nanoscopic water droplets. Another feature of these nanostructures is that hydrophobic labels (*e.g.* cholesterol, steroid drugs, or hydrophobic dyes (*e.g.* cyanine 7.0)) can be attached to the N-terminal ends of the peptide sequences. This allows the incorporation of hydrophobic drugs, or the formation of cholesterol-nanodomains, which can be used for the physical adsorption of hydrophobic drugs. Another strategy that has been successfully tested for the treatment of glioblastoma [25] is to extend the linear peptide sequences by up to 10 D, E, or S units, which are then used to bind hydrophilic drugs *via* esterase-cleavable bonds. This concept has been proven successful for treating glioblastoma cell cultures with perillyl alcohol [25].

3.9. Polymer-based Nanoparticles

Whereas the degree of protection of antibodies and peptides by inorganic nanoparticles is usually greater, polymer-based nanoparticles are much more flexible and can easily be tuned in molecular weight, particle size, and surface functionality [255]. Furthermore, both, targeting and therapeutic peptides can be easily attached to side chains in polymeric formulations. It should be noted that soft nanostructures may be able to spread out on a cell membrane, which would increase the strength of interaction with the latter and could trigger either membrane-integration, endosomal uptake or direct transport through the membrane [96].

As discussed above for supramolecular peptide aggregates and hydrogels, polymer-based nanoparticles are also divided in two groups: Nanostructures formed by means of non-covalent and covalent interaction. It should be noted that the boundaries between all delivery systems based on organic structures are somewhat blurred.

3.10. Polymer-based Nanoparticles Based on Noncovalent Interactions

Noncovalent complexation strategies are usually based on electrostatic interactions, although specific binding motifs, such as avidin-biotin, have already been successfully explored [256]. For instance, biotinylated poly(propylacrylic

acid) (PPAAc) and a biotinylated anti-CD3 antibody have been combined with streptavidin to form a ternary nanostructure, which was taken up by Jurkat lymphoma cells *via* receptor-mediated endocytosis [257]. It was speculated that endolysosomal release was facilitated by the PPAAc-"proton sponge". In a similar manner, poly(lactic-co-glycolic acid) (PLGA) based nanocarriers have been employed to protect anti-annexinA2 (AnxA2) antibody [258]. However, in this case the encapsulated antibody was much better retained by the formulation, resulting in the slow release of active antibody over 12 days.

3.11. Gene Transfection

Non-viral nanocarriers have been developed and investigated, due to side effects and toxicity of viral nanocarriers [250]. For a non-viral gene delivery to be efficient, the vector must target specific cell receptors, protect DNA from degradation, deliver DNA into the nucleus, and disrupt endosomal membranes, a major biological barrier faced in gene delivery approaches [250, 259]. For this approach, fusogenic peptides have been developed, which have gained attention as a promising gene delivery nanocarrier. Fusogenic peptides (FP) are virus- based amphiphilic peptides capable of interacting with phospholipid membranes for membrane fusion and/or lysis [259] (Fig. **7**). Among other, GALA, a 30 amino acid residue (WEAALAEALAEALAEHLAEALAE ALEALAA) and pH-sensitive peptide (see above), was synthesized and shown to be an effective membrane fusion peptide at pH 5. In order to further improve this peptide, due to anionic properties that limited the association with DNA, KALA and RALA, fusogenic peptides were developed, which in each case the glutamic acid was substituted with lysine or arginine, respectively [17]. However, Nouri *et al* constructed a recombinant biopolymer for each FP, in order to determine which peptide is more efficient for gene delivery [259]. In this study, membrane disruption, cell toxicity, and transfection experiments were conducted. Results demonstrated that biopolymer containing GALA FP peptide had lower cell toxicity, better ability to disrupt membranes, as

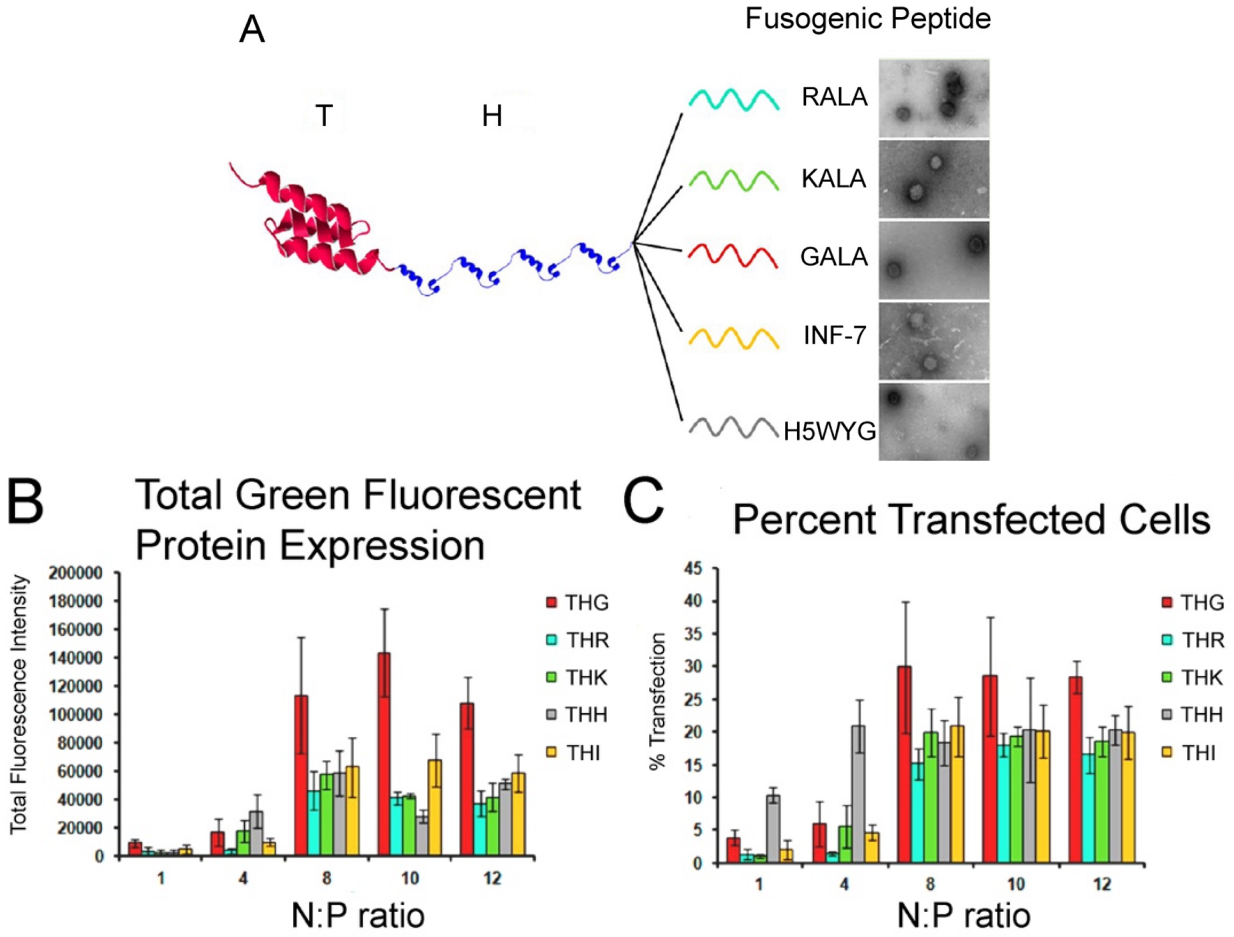

Fig. (7). (**A**) "Schematic representation of recombinant biopolymers composed of a targeting motif (T), four repeating units of histone H2A with an inherent nuclear localization signal (H) The 3D structures of T and one repeat of histone H2A are predicted by SWISS-MODEL program. (**B**) A bar chart that quantitatively demonstrates total green fluorescence intensity of transfected SKOV-3 cells with biopolymer/pEGFP complexes. (**C**) A bar chart that quantitatively demonstrates percent transfected cells with biopolymer/pEGFP complexes at different N:P ratios."Adapted with permission from (Nouri FS, Wang X, Dorrani M, Karjoo Z, Hatefi A. A Recombinant Biopolymeric Platform for Reliable Evaluation of the Activity of pH-Responsive Amphiphile Fusogenic Peptides. Biomacromolecules. 2013;14(6):2033-40. doi: 10.1021/bm400380s.) Copyright (2013) American Chemical Society [259]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

well as better transfection efficiencies compared to the rest [18]. Even though, GALA has limited association with DNA individually, once it is used to form a biopolymer, it has been shown to be a better suitable nanocarrier for gene delivery. In Fig. (**7**), the inherent nuclear localization signal (H) has the following sequence:MVDNKFNKEMRNAYWEIALL PNLNNQQKRAFITSLYDDPSQSAN LLAEKKLNDAQA PKGGGGSGGGGSGRKRSGRSKQGGKARAKAKTRSSR AGLQFPVGRVHRLLRKSGRGKQGGKARAKAKTRSSR AGLQFPVGRVHRLLRKSRGKQGGKARAKAKTRSSRA GLQFPVGRVHRLLRKSGRGKQGGKARAKAKTRSSRA GLQFPVGRVHRLLRKGGG). The fusogenic peptides used were (INF7 (GLFEAIEGFIENGWEGMIDGWYG), GALA (WEAALAEALAEALAEHLAEALAEALE ALAA), KALA (WEAKLAKALAKALAKHLAK ALAKALKAGEA), RAL A (WEARLARALA RALARHLARALARALRAGEA), and H5WYG (GLFHAIAHFIHGGWHGLIHGWYG)).

Polyethyleneimine (PEI) has been widely used as a delivery vector because it is positively charged at physiological pH and can form complexes with DNAs and RNAs. Effective endosomal escape is fast, because PEI is a "proton sponge" at endosomal pH, which destabilizes the endosomal membrane [260]. Similarly, PEI is used to associate with negatively charged proteins, such as anti-lamin antibodies. Nuclear lamins interact with membrane-associated proteins to form the nuclear lamina on the interior of the nuclear envelope [261]. In a similar approach, anti-synuclein antibody containing polybutylcyanoacrylate nanoparticles were taken up *via* low-density lipoprotein receptor mediated endocytosis.

3.12. Targeting Peptides to Improve Uptake and Delivery

Besides facilitating the transportation and delivery of payloads, peptides can also be used to develop a specific targeting nanocarrier. Therapeutic agents have been identified to be effective against pathogens, but most of the time they are toxic to healthy cells too, just as with chemotherapy. Therefore, the development of a vehicle that will be activated to release the therapeutic agents only after reaching the targeted tissue is crucial. Multiple nanocarriers have been developed with effective targeting mechanisms using peptides. Peptides sequences have been designed to target specific enzymes that play essential roles for pathogens to survive, which was effectively applied to deliver drugs to specific tissues, like brain tumors [247] This idea has been exploited to develop nanocarriers that could target challenging tissues, like bones. Jiang *et al*. developed a drug delivery system to target bone tissue exclusively. The nanocarrier was composed of PLGA-based nanoparticles linked to a fluorescent label poly-aspartic acid peptide sequence, which has been demonstrated to bind effectively to hydroxyapatite (a mineral found in bones) [247, 262]. For this study, *ex vivo* experiments were conducted to determine binding of nanoparticle to multiple tissues. Mouse tibia, brain, heart, liver, spleen, kidney, lung, and gastrointestinal tract tissues were exposed to poly-Asp nanoparticle, and results demonstrated that nanoparticle bound specifically to bone tissue [262]. It was found that the simple peptide sequence DDDDDDC was capable of targeting bone cells *via* binding to hydroxyapatite. This finding has the potential of leading to a very effective delivery for the (chemo) therapy of various bone cancers.

CONCLUSION

Therapeutic peptides, as well as antibody fragments and antibodies are constantly increasing in importance as components of smart therapeutics that can effectively target and treat diseases, [1, 3, 5, 31, 99, 111, 117, 168, 171]. Chemical strategies for enhancing the efficacies and/or circulation/residence times of peptides and proteins are straightforward. They comprise chemical derivatization, such as PEGylation [62], and the synthesis of retro-inverso peptides [165], which are much more stable against proteolytic degradation [2, 3]. Nanoparticle-based strategies work well in further decreasing proteolytic degradation due to an increase in size [1]. However, this can to be carefully counterbalanced with the clearance of nanoparticles from circulation by the reticuloendothelial system, because nanoscopic structures have the size of viruses (and larger structures of bacteria) [62]. Furthermore, they are known to trigger immune-responses, which increase their uptake even more [263]. When selecting the type of nanoformulation, six factors should be carefully considered: Which physiological barriers does the nanoformulation have to cross? What is the intended mechanism of cellular uptake (phagocytosis, pinocytosis, or non-specific uptake)? What level of protection does the payload require? Is immediate or timed release desired? What is the fate of the nanoformulation after the payload has been delivered? What off-target effects are anticipated? The answers to these questions will be the guide through the plethora of nanodelivery systems to an intelligent tailored solution for any drug delivery problem.

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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