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Phage-displayed peptides targeting specific tissues and organs

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ABSTRACT

Phage display is a powerful and widely used technique to find novel peptide ligands. A massive amount of peptide sequences have been identified for all kinds of materials, and peptides that may have targeting capabilities towards specific cells and tissues have received special attention in biomedical sciences. As a result, it is increasingly harder to follow all the work that has been done, which sometimes leads to many promising ligands receiving little attention, together with the publication of false positives that have already been found. The aim of this review is to provide an updated and comprehensive list of phage-displayed peptides targeting different tissues and organs. The limitations of the technique are carefully analysed and the future perspectives envisaged.

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Introduction

Nowadays, much research in the biomedical field is focussed in nanotechnology, which remains as a promising approach for overcoming the challenges of drug delivery [1]. Directing drugs to the site of disease and getting through biological barriers, thus improving specificity and efficiency of both treatments and detection agents, are of paramount importance for positive therapeutic outcomes [2]. Different approaches have been implemented, and many have delved into the discovery of targeting molecules able to reach specifically the diseased cells [3]. These molecules could either be bound to the drug or detection agent directly, or attached to the surface of nanocarriers. Peptides are the most typical targeting molecules, as they can be ligands of specific cell membrane receptors, improving intracellular delivery of drugs across biological barriers. For example, transferrin-like ligands can promote passage through the blood–brain barrier (BBB) via receptor-mediated transport [4]. Tumour-homing motifs can also be found, such as the integrin-binding RGD and the CD13 aminopeptidase-binding NGR [3]. Furthermore, cell-penetrating peptides can cross the cell membrane, enabling the treatment of intracellular disease targets. This process is suspected to occur through endocytosis or direct penetration, depending on the peptide sequence and the substance they are conjugated to [5]. Phage display is one of the main tools for identifying novel targeting peptides [6]. The number of homing motifs keeps increasing, so it is important to critically list them and review the work that has been put into this field.

Targeted tissue delivery of therapeutic and diagnostic nanocarriers provides several advantages, including the reduced side effects of drugs, the possibility to overcome drug resistance and the ability to administer lower doses while still achieving a therapeutic effect. Currently assumed targeting mechanisms can be divided in two categories: passive targeting and active targeting. In the former, nanoparticles avoid the immune and reticuloendothelial systems due to their specific properties, such as size, shape, composition and surface charge. In active targeting, the functionalisation of the nanoparticle surface with ligands able to recognise specific molecules expressed on the target cells or tissues enhances their accumulation at a specific site, reducing off-target side effects. Appropriate ligands attached to the surface of nanoparticles for active targeting include proteins (transferrin, antibodies), vitamins (folic acid), aptamers (RNA) and, of course, a myriad of peptides.

To date, phage display is one of the most common methods for the identification of specific peptide ligands, and is already widely utilised for enhanced active targeting of nanocarriers, as described in the section 'Organ and tissue targeting peptides' of the present review. Understandably, great effort has been directed towards the identification of peptides targeting cancerous cells. Nevertheless, this approach presents some disadvantages. Tumour cell lines that are commonly used in *in vitro* studies may have important differences with the cells from the actual tumours, and each kind of cancer contains different surface receptors and antigens. Thus, homing peptides may lack targeting capabilities *in vivo*, and each peptide is likely to be specific for a certain cancer cell, limiting its clinical usefulness, as a different formulation may be required for different tumour cells affecting the same organ. Some examples can be the liver, which can be affected by hepatocellular carcinoma, cholangiocarcinoma or metastatic adenocarcinomas with different immunohistochemical profiles [7]; the pancreas, which can suffer from cancer developed from different histological precursors, pancreatic neuroendocrine tumours or lymphomas [8]; and the brain, because various glioma types exist [9]. Moreover, targeted drug delivery cannot work unless the nanocarriers are able to reach the site of the tumour and effectively cross biological barriers [2]. These barriers, depending on the delivery of the drug, can be endothelia (intravenous), the gastrointestinal barrier (oral), the air–blood lung barrier (nasal or aerosols) or the skin (topic). The endothelium is the barrier that needs to be dealt with most of the time, as intravenous administration is by far the most popular and effective for targeted nanoparticles.

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Unlike cancer cells, endothelial cells present less variability between different tumours. Hence, nanoparticle accumulation in specific tissues and organs can be promoted by aiming for specific endothelial cells. This way, it is possible to both target the site of interest and enable the nanoparticles to cross biological barriers. This strategy might also allow using the same targeting peptides for different diseases affecting the same organs, making the use of *in vivo* phage display followed by *in vitro* tests on endothelial cell cultures very valuable. During the last two decades, *in vivo* phage display has received growing attention, as presented by Babickova and colleagues in a detailed review on the possibilities that this technology presents [10]. In this technique, phage libraries are intravenously injected in living mice, rats or even humans [11], and phage are recovered from the tissue of interest. The selection process is stronger, occurring in physiological conditions and the resulting targeting peptides have higher probability of clinical translation, being suitable to be tested on human biopsies or cells. This technique has been applied by many with the aim of determining homing peptide motifs in different organs and tissues, therefore 'mapping' the vasculature or, in other words, creating an address book of different endothelia [12–16].

Peptides can easily be conjugated to a great variety of molecules, conferring targeting properties to drugs, whole proteins or oligonucleotides. Thus, they can be applied in drug delivery, imaging, diagnosis, and gene therapy. Besides, nanoparticles of different nature have been functionalised with homing peptides, including liposomes, colloidal and polymeric entities [17]. A different noteworthy strategy is to employ the phage itself as carrier for therapeutic or detection agents [18]. This principle has been implemented in adenovirus-based gene delivery vectors too, even generating adenovirus-based peptide libraries [19], but it is not covered in this review. Nevertheless, clinical translation of targeting peptides is limited, and most phage display-derived drugs that have been approved or are currently undergoing clinical trials are antibodies [20].

Limitations of phage display and targeted delivery

Unfortunately, inconveniences and limitations of phage display are often overlooked. First of all, it is of paramount importance to choose phage display strategies with translation to clinic in mind. On the one hand, experiments based solely on *in vitro* panning using cell lines may not be enough, as selected peptides could behave differently *in vivo*, showing unexpected binding or accumulation patterns. On the other hand, *in vivo* phage display is mostly done with animal models, which may poorly represent the investigated condition. For instance, differences in hemorheology and hemodynamics are not fully understood yet, and may affect binding efficacy of vascular-targeted entities [21], and pathological features of neurodegenerative disease models are not identical in humans [22]. Besides, this technique might lead to the selection of species-specific ligands that would not have any targeting properties in humans. If a given peptide selected in mice were a ligand of a membrane receptor that is not present or has a different binding site in humans, that peptide would lack clinical relevance. In fact, the binding mechanisms and receptors involved often remain unknown, and meticulous work is required to elucidate how the peptides and their targets behave at the molecular level.

Secondly, phage display can be biased, and it is common to come across target unrelated peptides (TUPs) [23–26]. These motifs do not bind the actual target, but other elements in the system, mostly the polystyrene of which common labware is made, bovine serum albumin, streptavidin, antibodies and bivalent metal ions. Sometimes, TUPs are not selected because of a non-desired binding, but because certain peptide sequences confer propagation advantages to the virus, creating phage clones able to replicate faster than others during the amplification step between phage display rounds, and thus producing a 'false' enrichment. In addition, biological biases can compromise the integrity of the library, as some amino acids may be over-represented, and mutations and recombination may also take place. The Biopanning Data Bank [27] (BDB, http://immunet.cn/bdb/index.php) is an outstanding tool to minimise the false positives, as it is possible to check whether a particular peptide has previously been found in other studies with unrelated targets, in which case it would most likely be a TUP. Although some peptides may specifically bind to different targets, probability of a given peptide sequence being selected in different unrelated experiments from a random library with a 10^{8-9} diversity is minimal, if not negligible.

The HIAYPRH peptide (Table 4) is an illustrative example of a TUP that somehow remains overlooked by numerous research groups up until now. It was first reported by Lee [28] in 2001 as a TfR ligand but in 2007 Brammer and co-workers [29] demonstrated that it was actually a TUP and later on it was also listed as a TUP in some reviews on this topic [24–26]. Furthermore, 30 entries can be found in the BDB for 21 different targets, clearly supporting that it is indeed a TUP. Nevertheless, several articles can be found where HAIYPRH is still used for its wrongly attributed targeting capabilities, the two most recent at the time of writing this review having been published in 2017. More peptides likely to be TUPs are made bold in the tables, because each one of them was found to be in several data sets referring to different targets in the BDB. Thus, it is clear that many investigators are unaware of the importance of TUPs, leading to unreliable scientific results. In fact, interactions between nanoparticles, peptides and targets, and binding and internalisation mechanisms are yet to be properly explained, so the interpretation of the results may be questionable in many cases, such as when favourable nanoparticle targeting has been described using TUPs.

In addition, even though novel imaginative drug delivery systems are abundant, effective drug delivery remains a challenge. Currently, presence of homing peptides is scarce in the clinic due to various reasons. For example, stability of the peptides can easily be compromised upon entering the body, or the newly conjugated peptide may not retain the same conformation as in the phage. Also, clinical translation of nanoparticle-based treatments is far from trivial. Chan and colleagues thoroughly discussed the hindrances of this technology in cancer therapy, and they showed that progress in this field is slower than expected. Most of the described obstacles are not restricted to cancer treatment, as any nanocarrier must face the mononuclear phagocytic system, renal clearance, flow and shear forces, aggregation and the formation of a protein corona [30]. Therefore, reaching the target cells with the nanoparticles is troublesome. On top of that, binding specifically to the correct endothelial cells by the homing peptides may not guarantee the delivery of the drug into the diseased cells. Nanoparticles still need to be transported across the endothelium, be taken up by the target cells, and the drug must be released after the whole process [2,3]. Even with a favourable biodistribution, pharmacokinetics are yet hard to elucidate, and the efficiency of the treatment is often low. For this reason, relatively high doses are usual in animal model studies, which in turn impose hardships in the scaling-up, as it is complicated to produce big amount of nanoparticles with no harm to stability and shelf-life, while also

Table 1. Peptides targeting endothelial receptors, the heart and atherosclerotic plaques.

Peptide sequence	Target	Used animals and cells	Conjugated to	References
SIGYPLP	Endothelium	HUVEC	Adenovirus	[37]
LSIPPKA FQTPPQL LTPATAI	LOX-1 endothelial receptor associated with hypertension and atherogenesis	LOX-1 overexpressing hepG2		[38]
CNIWGVVLSWIGVFPEC	Restenotic plaques	Vascular smooth muscle cells ApoE$^{-/-}$ mice		[39]
NTTTH	Inflamed endothelia (liver and kidneys)	BALB/c mice HUVEC, HMVEC	EGFP	[40]
VHPKQHR (tetramer)	VCAM-1, associated with inflammation	ApoE$^{-/-}$ mice	^{18}F, Cy5	[41]
		MHEC	Polyelectrolyte PEG-K30 micelles	[42]
CRKRLDRNC	IL-4 R, atherosclerotic plaques	Ldlr$^{-/-}$ and ApoE$^{-/-}$ mice	Fluorescein, ^{111}In	[43]
CRTLTVRKC	Stabilin-2, atherosclerotic plaques	BAEC, primary human atherosclerotic tissues	Glycol-chitosan-cholanic acid NPs and Cy5	[44] [45]
CLWTVGGGC	Atherosclerotic plaques, TNF-alpha activated endothelial cells	Ldlr$^{-/-}$ mice BAEC (only binding) C57BL6 mice BALB/c mice HUVEC	Fluorescein	[46]
QPWLEQAYYSTF	Normal endothelium		Biotin	[47]
YPHIDSLGHWRR	Hypoxic endothelium		FITC	[48]
LLADTTHHRPWT **SAHGTSTGVPWP**				
VPWMEPAYQRFL TLPWLEESYWRP	Normal and hypoxic endothelium			
HWRR	GRP78 in ischaemic endothelium			[49]
CSTSMLKAC	Ischemic heart	Sprague–Dawley rats	Sumo, mCherry	[50]
DDTRHWG	Heart	WKY and SHRSP rats RGE, Y-PEN rat EC, hEC	Adenovirus	[51]
CARPAR	Heart. EST	BALB/c, FVB, C57BL/6 mice	Fluorescein	[52]
CKRAVR	Heart. Sigirr, TIR8	HCAEC, HUVEC		
CRSTRANPC	Heart. MpcII-3			
CPKTRRVPC	Heart. bc10	BALB/c, FVB, C57BL/6 mice	Fluorescein	[52]
CSGMARTKC		WKY and SHRSP rats	Gp91ds peptide	[53]
CRPPR	Heart. CRIP2, HLP, ESP-1	HCAEC, HUVEC		

Bold values: sequences likely to be target unrelated peptides (TUPs, see 'Limitations' section).

maintaining reasonable manufacturing costs. In fact, nanoparticle design and synthesis are of foremost relevance, because shape, size and zeta-potential greatly affect the efficacy [31,32].

Organ and tissue targeting peptides

In spite of the numerous challenges, a great variety of targeted nanocarriers have been produced during the last couple of decades and promising formulations can still be found. Liposomes are the most abundant of the clinically approved nanomedicines, but significant progress is being made towards stimuli–responsive systems for controlled drug release and active targeting mechanisms [33]. Anselmo and Mitragotri reviewed the state of nanoparticles in the clinic [34]. Here, we aim to list targeting peptides for various organs and tissues, especially endothelia, obtained by different phage display experiments. As mentioned previously, functionalising nanoparticles with tissue- or organ-specific targeting peptides can be crucial when aiming to overcome biological barriers, as nanocarriers are of no use if they are not able to accumulate in the site of disease. Whenever possible, an overview on the progress achieved for a particular peptide was provided in order to evaluate the effectiveness of the sequence, referencing different articles in chronological order. Possible TUPs were also highlighted, which are proven to lack any targeting capabilities and should not be used in future studies.

Vascular system

Intravenous injection being the predominant form of nanocarrier administration, many have pursued the treatment of cardiovascular diseases like atherosclerosis [35] and ischemia [36]. In this case, elements of the circulatory system are the target of *in vivo* phage-displayed peptides, such as the heart, atheroma plaques, inflammation sites and ischaemic tissues, as shown in Table 1. In the beginning, only cell cultures were used for phage panning, and further development was not pursued. These peptides might present issues in an *in vivo* setting, but combined *in vitro* and *in vivo* phage display followed soon. Various animal models have been used, both mice and rats, while the predominant cells are human umbilical vein endothelial cells (HUVEC). These cells have been used aiming to target the heart in general, ischaemic endothelia, and inflamed endothelia in the liver and kidneys, as cellular models are usually limited. Using essentially the same kind of panning while looking for different peptides raises questions about the specificity, so most researchers opted to strengthen the selection process combining it with *in vivo* phage display. In fact, 20 out of the 25 peptides listed in Table 1 were *in vivo* phage-displayed peptides that were also tested *in vitro*. However, this does not always prevent the appearance of TUPs such as LLADTTHHRPWT and SAHGTSTGVPWP. These false positives are relatively common because receptors remain unknown for the vast majority of phage-displayed peptides, as usually scientists are satisfied with appropriate biodistribution or colocalisation imaging studies, reporting specificity only towards certain cells, tissues or even whole organs. Nevertheless, some groups were able to find the receptor molecules for the peptides, which makes more accurate and precise experiments possible, and their results more reliable, although not infallible, as CRPPR is another TUP. On the contrary, CRKRLDRNC and CRTLTVRKC have a high chance of being specific atherosclerotic plaque targeting peptides, as they have been tested in two different mouse models, bovine aortic

Table 2. Peptides targeting the pancreas.

Peptide sequence	Target	Used animals and cells	Conjugated to	References
CRVASVLPC	Pancreas endothelium. PRLR	C57BL/6 mice PRLR overexpressing COS-1		[54]
SWCEPGWCR	Exocrine pancreas and islets. (Uterus vasculature too?)	BALB/c mice		[55]
LSGTPERSGQAVKVKLKAIP	β-cells in islets	Sprague–Dawley rats		[56]
CHVLWSTRC	Ephrin A2 and A4 receptors in pancreas islet vessels	C57BL/6 and NOD mice	PLGA-PEG NPs	[57]
CVSNPRWKC		Murine CE cells	PEG-p(CBA-DAH)	[58]
		MS1 cells		[59] [60]
LSALPRT	Islet cells	Sprague–Dawley rats	TAMRA	[61]

Table 3. Peptides targeting the kidneys.

Peptide sequence	Target	Used animals and cells	Conjugated to	References
CLPVASC	Glomeruli and tubules	BALB/c mice		[63]
ELRGD(R/M)AX(W/L)	Basolateral side of cortical collecting ducts	Sprague–Dawley rats		[64]
GV(K/R)GX_3(T/S) RDXR	Proximal convoluted tubules	Sprague–Dawley rats		[65]
HITSLLS HTTHREP	Tubule and glomeruli endothelium	WKY rats	Adenovirus	[66]
ANTPCGPYTHDCPVKR	Kidney	Kunming mice	Captopril FITC	[67]

endothelial cells (BAEC) and human atherosclerotic tissues, not only binding known receptors, but also showing the potential to work *in vivo* and in human cells. Besides, while most studies were limited to the detection of the binding using mostly fluorescent probes, those two peptides where also successfully attached to chitosan nanoparticles. Unfortunately, no more work has been published on these peptides since 2010 [47–49].

Pancreas

The pancreas also became a target for phage display, mainly the islets where beta-cells reside (Table 2). Their abundance is remarkably reduced in both types I and II diabetes, so an accurate targeting method would allow for improved diagnosis, assessment and treatment of diabetes [62]. The progress in this area is yet limited, and relatively little research has been done for most sequences. A single *in vivo* phage display, for instance, is not sufficient evidence to justify pancreas targeting. In fact, SWCEPGWCR may also bind to the endothelium of the uterus, so its specificity is compromised, as opposed to CHVLWSTRC and CVSNPRWKC, which seem to be reliable. They have been selected using both murine and cellular models, were proven to bind to Ephrin A2 and A4 receptors in the islet vessels, and were successfully conjugated to PEG and PLGA nanoparticles. These functionalised nanoparticles are a promising approach to efficiently reach the pancreas islets, and the latter are efficient drug encapsulating agents, due to their hydrophobic core.

Kidney

Table 3 lists a few examples of kidney homing peptides, although they are relatively scarce too. Further basic research would be required for a better understanding of the surface receptors of the diverse cell types in such complex units as nephrons. In addition, kidney targeting receives little consideration due to their excreting function. *In vivo*, renal clearance is one of the major impairments for nanoparticle targeting, which can frequently accumulate in the kidneys, and get excreted if the hydrodynamic diameter is smaller than 5.5 nm [30]. It is therefore arguably easy to reach them, but characterising specific interactions and validating *in vivo* data is far from trivial. A similar reasoning could be applied to the liver, for which no phage-displayed peptides have been reported, as high proportions of virtually every nanoparticle accumulate in this organ. All the sequences here reported that are supposed to target the kidneys where selected in a single *in vivo* phage display experiment, so their potential for clinical translation is yet to be investigated.

Brain

In contrast, considerable attention has been paid to the brain microvasculature, owing to the blood–brain barrier (BBB). The tight junctions and efflux pumps in this endothelium greatly reduce its permeability, and drug delivery to the brain is severely impaired. Table 4 shows that plenty of homing peptides have been described, and all kinds of imaging agents and nanoparticles have been employed, treatment of Alzheimer's disease being one of the main driving factors of all this work. Some promising homing peptide are collected here, even though clinical trials have not been reached yet. Receptor mediated transport is thought to be the most feasible way to cross the BBB, without transiently impairing its function. To this end, many research groups have focussed in the well-known transferrin receptor (TfR) and the discovery of transferrin-like ligands that are able to undergo transcytosis. Several phage display experiments have been conducted which led to novel ligands, the capabilities of which are still being studied after more than a decade from the first publication. Nevertheless, the fact that many publications exist about a given homing peptide does not guarantee its reliability. As mentioned before, HAIYPRH has been demonstrated to be a TUP. THRPPMWSPVWP is a much more dependable sequence, which has been used in all kinds of conditions and nanoparticles, and has been demonstrated to work in human TfR positive cells. In fact, this sequence is, to date, one of the most promising candidates for clinical translation.

Even more peptides have been found by *in vivo* phage display, where Sprague–Dawley rats have been widely used. Regrettably, the specific targeted receptor has only been determined in two cases: the CMPRLRGC sequence is a ligand for the LDL receptor, and CRTIGPSVC for Apo transferrin. In many cases, mouse brain endothelial bEnd.3 cells have also been used for panning, imaging and *in vitro* BBB crossing experiments. The TGNYKALHPHNG peptide provided good results in various complex studies in mice, even in an

Table 4. Brain homing peptides.

Sequence	Target	Used animals and cells	Conjugated to	References
CLSSRLDAC	Brain	BALB/c mice		[63]
GHKAKGPRK	hTfR (BBB)	hTfR + HEK293, CHO, T24	Adenovirus	[68]
		hBME	(C-Stp4)2-K-PEG-	[69]
		DU-145, N2A	-PEG-STP	[70]
HAIYPRH	hTfR (BBB)	hTfR + CEF	GFP	[28,71]
		Sprague–Dawley rats	PEG-Liposomes	[72]
		ICR and BALB/c mice	PANAM-PEG	[73,74]
		BCEC, Bel-7402, NCI-H1299	bPEI	
THRPPMWSPVWP	hTfR (BBB)	hTfR + CEF, U87MG, HT29, NCI-H1299	GFP	[71]
		BCEC, BMVEC, brain glioma cells	Ga-68	[75]
		BALB/c mice, Sprague–Dawley rats	AuNPs	[76]
			bPEI	[73]
			PEG-Liposomes	[77]
HLNILSTLWKYRC	GM1	Sprague–Dawley rat primary motor neurons	Fluorescein	[78]
	Monosialotetrahexosyl-ganglioside	and dorsal root ganglion	PEI	[79]
		PC12, HEK293	PEG-b-PCL	[80]
				[81]
CAGALCY	Brain microvasculature	BALB/c, FVN/N, C57BL mice	GST	[82]
			AgNPs	[83]
CLEVSRKNC	Ischemic brain, apoptotic neurons	Sprague–Dawley rats, ICR mice	Fluorescein, ^{131}I	[84]
		BCEC	Liposomes	[74]
RPRTRLHTHRNR	Aβ(1-42)	(APPswe/PS1)E9 and HuPS1A246E mice	FITC	[85]
(D-aa)	across the BBB	C57BL/6 mice	FAM	[86]
		PC-12	^{3}H	[87]
		RBMEC/rat astrocyte co-culture		[88]
ACTTPHAWLCG	Nose to brain	Wistar rats		[89]
GLAHSFSDFARDFV	Brain endothelium	C57BL/6 mice	Liposomes	[90]
GYRPVHNIRGHWAPG		hCMEC/D3		[91]
TGNYKALHPHNG	Brain, across the BBB	Nude, ICR and BALB/c mice	PEG-PLGA NPs	[92]
		BCEC, bEnd.3	PEG-PDMAEMA	[95]
			PEG-PLA	[93]
				[94]
CRTIGPSVC	Apo transferrin	Nude and BALB/c mice	Adenovirus	[95]
		U87MG, hTfR + rat glioblastoma 9L cells	PEG-PLA	[96]
		bEnd.3		
CTSTSAPYC	Brain	ICR mice		[97]
CSYTSSTMC	Brain	Sprague-Dawley rats		[98]
CMPRLRGC	hLDLR (BBB)	C57BL/6 mice	Rhodamine	[99]
		Wistar and Sprague–Dawley rats	Fluorescent peptide	[100]
		hLDLR + CHO, BMEC	h-IgG1 Fc	[101]
TPSYDTYAAELR	Brain across BCSFB	Sprague-Dawley rats	FITC	[102]
RLSSVDSDLSGC	CSF transport	Wistar rats	Biotin, Streptavidin	[103]
	(BBB/BCSFB)		BACE1 peptide	
CAQK	Acute traumatic injury	BL6 mice	FAM, PEG-Ag NPs	[104]
		Human brain tissue	Porous silicon NPs	
SGVYKVAYDWQH	Brain endothelium	Human BBB model, bEnd.3	GFP, Rhodamine	[105]

Bold values: sequence likely to be a TUP.

Alzheimer's model where it was used to target drug-loaded polymeric nanoparticles [92–94,106]. However, proving the ability to bind to human cells is determinant for clinical relevance, which, to date, has not been achieved for this peptide. The BBB model based on hCMEC/D3 seed on a Transwell has become quite popular for this reason. Nevertheless, the *in vivo* step is still crucial: Díaz-Perlas et al. [105] used human and murine cells to select a single peptide, SGVYKVAYDWQH, which is another TUP that has also been selected in non-related experiments. Sometimes, isolated phage-displayed peptides failing to work *in vitro* may not be due to the inadequacy of the phage display, but because the targeting entity was not only the randomised sequence. Rooy et al. [90,91] demonstrated that the ability to bind brain cells was significantly enhanced when the two selected peptides where synthesised together with part of the original phage coat protein, as the conformation they adopted within the phage was vital for the process.

Other approaches to circumvent the BBB such as nasal administration or cerebrospinal fluid (CSF) targeting have been less investigated. Some drugs and virus can be transported through the olfactory pathway after intranasal administration, but to the best of our knowledge, only Wan and co-workers have explored this route by phage display [89]. The CSF passage takes advantage of the influx of this fluid into the brain parenchyma, postulating that drugs could be transported by that influx once they reached the CSF, even though little is known on the specifics of this mechanism [103,107]. The RLSSVDSDLSGC peptide is thought to be transported this way, and even though only Wistar rats were used, this phage display study was especially thorough in terms of phage administration, isolation and sequencing. Another example of a thorough study is the one conducted by Mann and colleagues, where the short CAQK motif was found to target traumatic injuries in the brain. In this article, *in vivo* and *in vitro* tests are reported, the latter using actual human brain tissues instead of the hCMEC/D3 cell line. Although BBB crossing cannot be assessed this way, immortalised cells may induce some kind of bias, whereas ex vivo tests possess high physiological relevance. Moreover, the researchers reported a strong sequencing methodology, using both Sanger and Next Generation Sequencing, and conjugated the peptide to nanoparticles and antibodies in order to demonstrate its targeting properties.

Table 5. Peptides homing to the lungs.

Sequence	Target	Used animals and cells	Conjugated to	References
CGFELETC	Alveolar capillaries.	BALB/c mice	PEG-coated	[55]
CGFECVRQCPERC	Membrane dipeptidase (MDP)	MDP in COS-1	ZnS-capped	[108]
		LE cells	CdSe Qdots	[109]
			IFNalpha2a	[110]
QPFMQCLCLIYDASC	Alveolar epithelium	BALB/c mice	FITC	[111]
RNVPPIFNDVYWIAF		A549 LE cell line (ATII)		
VNTANST	Lung endothelium	WKY rats	Adenovirus	[112]
CTSGTHPRC	Alveolar epithelium	Primary type II rat alveolar epithelial cells	PANAM G5.5 dendrimer	[113]
SGEWVIKEARGWKHW-VFYSCCPTTPYLDITYH	Epithelium. nAChR-a1	CrljOri:CD1 (ICR) mice	Alexa-488	[114]
		MLE12, C2C12	Cy-5.5	

Bold values: a sequence likely to be a TUP.

Table 6. Intestine homing peptides.

Peptide sequence	Target	Used animals and cells	Conjugation	References
YSGKWGW	Intestine (intravenous injection)	BALB/c mice		[55]
LETTCASLCYPS	Peyers patches	Wistar rats, IEC-6	Biotin	[115]
YQCSYTMPHPPV		Human Peyer's patch tissue sections	Adsorbed to streptavidin-polystyrene particles	
VPPHPMTYSCQY		Caco-2		
YPRLLTP	Transmucosal transport, recovered in spleen	Lewis rats		[116]
CSQSHPRHC	Inflammatory bowel	C57BL/6Ncrj mice		[117]
CSKSSDYQC	Villi lamina propria, epithelium goblet cells	Sprague-Dawley rats	Human growth hormone	[118]
		Caco-2/Raji B co-culture		[119]
				[120]
CKSTHPLSC	Peyer patch M cells, follicle associate epithelium	Sprague-Dawley rats	Biotin	[119]
		Caco-2/Raji B co-culture	Chitosan NPs, Alexa-488	
CTGKSC	M cells	Caco-2/Raji B co-culture	PCL-PEG NPs	[121]
LRVG			PLGA-PEG NPs	
SFKPSGLPAQSL	Intestine (intravenous injection)	BALB/c mice		[122]
		Human intestinal segments		
CTANSSAQC	Intestine (direct injection)	Sheep	Biotin, Streptavidin	[123]
		BALB/c mice	FITC, ^{125}I	

Lung

In Table 5, lung targeting peptides are reported. Two peptides bearing the GFE motif were proven to bind the membrane dipeptidase in alveolar capillaries, backed by significant evidence from both *in vivo* and *in vitro* studies. Interestingly, it has also been attempted to target lung epithelia, departing from the intravenous administration. Most experiments were done *in vitro* or *ex vivo*, but Wu et al. carried out an *in vivo* phage display with intratracheal instillation [108]. Unfortunately, one of the two isolated peptides, RNVPPIFNDVYWIAF, is a TUP. Overall, little advancements have been made investigating the lung barrier by phage display.

Intestine

Table 6 summarises intestine homing peptides. Same as in the case of the lung, most phage display experiments were meant to target the epithelium. Although intravenous drug administration is usually the most convenient in a scientific context, it suffers from limitations for human treatment, such as patient discomfort, an increased risk of infection in the sites of repeated injections and the risk of adverse effects resulting from rapid accumulation of high concentrations of drug. In this aspect, oral delivery is desirable, but also extremely challenging, due to the presence of numerous biological barriers [125,126]. Protein and oligonucleotide based drugs are degraded in the gut. If protective mechanisms are used to avoid this, it is still necessary to cross the mucosa and microbiota in the intestine before reaching the epithelium. Nonetheless, as more complex formulations give the chance for a successful oral delivery, targeting peptides to promote internalisation are still looked upon. Most of the phage display experiments were done panning directly against the intestinal tissue or *in vitro*. These peptides are very unlikely to reach the intestinal epithelium on their own, and no *in vivo* studies were conducted. Duerr and colleagues performed and *in vivo* phage display with gavage administration and recovered phage from the spleen, arguing that those clones had the ability to cross the intestinal barrier [116]. However, later Hamzeh et al. demonstrated that a proportion M13 phage is able to get into the bloodstream regardless of the variable sequence [124]. Therefore, selecting clinically relevant homing peptides to the intestinal epithelium does not seem feasible with these strategies, and might be better achieved targeting known specific receptors. For now, peptides are restricted to intravenous administration and the intestine is better targeted through the endothelium.

Others

Few homing peptides have been identified for other targets. In Table 7, it can be observed that many types of tissues have been explored. For instance, Rothenfluh and co-workers selected collagen binding peptides, and functionalised poly(propylene sulphide) (PPS) nanoparticles which were administered via intra-articular injection [127]. Another unusual application was presented by Surovtseva and colleagues, where the prestin protein in the cochlea was targeted, providing new insights on the hearing loss associated to outer hair cells [128]. The main drawback of this uncommon targets is that the wider scientific community shows little interest towards them, and they are forgotten once they are published, clinical translation being unlikely.

Real promises of phage display technology

Phage display was shown to be a powerful technique for the identification of homing peptides to virtually any target. Nevertheless, it has

Table 7. Examples of peptides targeting various organs and tissues.

Peptide sequence	Target	Used animals and cells	Conjugated to	References
LMLPRAD	Adrenal gland	BALB/c mice		[55]
CSCFRDVCC	Retina			
CRDVVSVIC	Retina			
CVALCREACGEGC	Skin hypodermal blood vasculature			
GLSGGRS	Uterus			
WYRGRL	Articular cartilage. Collagen II a1	Bovine cartillage grafts C57BL/6 mice	PPS	[127]
CPGPEGAGC	Breast vasculature. Aminopeptidase P	ICR CD-1 and MMTV PyMT mice		[129]
SMSIARL	Prostate	CD-1 mice		[130]
VSFLEYR		Human prostate tissue		
GPEDTSRAPENQQKTGC	Skin Langerhans cells	XS52 BALB/c mice	Biotin Liposomes	[131]
CKGGRAKDC	White fat vasculature. Prohibitin	C57BL/6 mice	FITC $(KLAKLAK)_2$	[132]
CARSKNKDC	Wound	Sprague-Dawley rats BALB/c mice CHO-K	Fluorescein	[133]
CHAQGSAEC	Thymus vessels	BALB/c mice		[134]
LEPRWGFGWWLK	Ear, cochlea outer hair cells. Prestin	Prestin + CHO and Cos-7	PEG-PCL	[128]
LSTHTTESRSMV		P7-p10 rats		
ACSTEALRHCGGGS	Retina abnormal neovessels	Sprague-Dawley rats		[135]
ASSLNIA	Muscle fibres	BALB/c mice C2C12		[136]

Bold values: a sequence likely to be a TUP.

been shown that important limitations exist, and the results can often be biased. TUP selection is clearly the main issue hindering phage display. From the peptides collected here, it can safely be concluded that the most promising sequences are always the ones that have gone through diverse phage displays. An experiment using a single cell line is prone to lead to non-specific peptides, for instance polystyrene-binding TUPs. Ligands can be much more reliable if a variety of appropriate negative controls are reported, such as phage displays on empty wells, different cells and proteins present in the media. The best way to get sequences with actual targeting capabilities is combining *in vivo* and *in vitro* phage display, ideally using different animal and cellular models. When the *in vivo* part is solid, the focus should be shifted towards proving that those peptides could be able to work in humans, as translation to clinic must be the final goal. In this step, primary cells and human tissue samples should be favoured, keeping the targets as close to the *in vivo* setting as possible. *In vitro* models consisting of cell lines can also be extremely valuable, such as in the case of BBB models based on Transwel® cultures, where the ability to cross the barrier can be evaluated. This can be achieved more easily when different researchers keep collecting evidence on the same peptides. However, performing innumerable phage displays is not the ultimate solution, as phage propagation related TUPs are not only unaffected by this, but also actively selected, due to the fact that they are more likely to arise the more the phages are amplified. Therefore, the most efficient way to get rid of TUPs is awareness. When a ligand is isolated, checking if it has already been reported by others is the first and most important task, so uploading data to the BDB is vital, as well as reading reviews listing known TUPs. In short, a good homing peptide is characterised by being the result of varied phage displays and not having been selected for unrelated targets.

For the time being, peptide selection and nanoparticle delivery are restricted to intravenous administration, as the oral route poses too many hardships, and other ways such as the skin or the alveolar epithelium have barely been explored. Hence, endothelial cells are the prime target for these homing peptides. Other types of cells, including tumour cells, may only be affected by peptides if the nanocarriers are already able to reach said cells when injected *in vivo*. Moreover, phage display targets must be looked upon by the wider scientific community, as it has been shown that the most promising targeting peptides are those on which more work has been done. This means that somehow 'unorthodox' targets where few people are working on are unlikely to get effective ligands. The BBB and the circulatory system itself have received the most attention, so disorders such as Alzheimer's disease and atherosclerosis have the highest probability to get treatments based on targeted nanocarrier delivery. Alzheimer's treatment in particular seems to be headed towards PEGylated liposomes or PLGA nanoparticles functionalised with BBB targeting peptides and loaded with Aβ plaque degrading agents. Ultimately, keeping track of the numerous achievements in the field is crucial, identifying TUPs, further developing previously discovered peptides and building up on the extensive work that has already been done in phage display.

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