Challenges and opportunities for non-antibody scaffold drugs

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The first candidates from the promising class of small non-antibody protein scaffolds are now moving into clinical development and practice. Challenges remain, and scaffolds will need to be further tailored toward applications where they provide real advantages over established therapeutics to succeed in a rapidly evolving drug development landscape.

Introduction

Monoclonal antibodies represent a well-validated and rapidly growing class of human therapeutics, characterized by a fully human nature, long serum half-life, bivalency and immune effector functions [1,2]. Despite their successes, monoclonal antibodies nevertheless face a range of restrictions that limit their applicability. Many of these restrictions relate to the size of the antibody molecule, which, in the case of the commonly used IgG isotype, is in the range of 150 kDa. Moreover, antibodies are complex multi-domain proteins, consisting of two different chains (heavy and light) and a total of six different domains (designated V_H, V_L, C_H1, C_H2, C_H3 and C_L). Assembly of the antibody molecule and much of its stability rely on the correct formation of disulfide linkages and on post-translational glycosylation of the constant region [3]. In combination, these requirements complicate the expression of human antibodies in general, and hinder the production in the cytoplasm of bacteria in particular [4,5]. To overcome the size and stability limitations of monoclonal antibodies, a large body of work has focused on the generation of small non-antibody scaffolds for human therapy and imaging applications [6–8]. In this review, we discuss challenges in translating such scaffolds into validated drugs, and provide updates on their use in clinical development and practice.

Challenges in the development of non-antibody scaffolds

Non-antibody scaffolds under active development broadly fall into two structural classes, namely domain-sized compounds (at 6–20 kDa molecular weight) and constrained peptides (2–4 kDa) (Fig. 1). Domain-sized scaffolds include Affibodies, Affilins, Anticalins, Atrimers, DARPins, FN3 scaffolds (e.g. Adnectins and Centyrins), Fynomers, Kunitz domains, Pronectins and OBodies, whereas Avimers, bicyclic peptides and Cys-knots are peptide-related. A large number of candidates derived from these scaffolds are currently under academic, preclinical and clinical development (Table 1) and have shown great potential in terms of affinity, target neutralization and stability, see [7] for a comprehensive review.

Despite the considerable promise of non-antibody scaffolds, conversion of the many examples of such modalities into differentiated drugs has been challenging. Although several candidates have progressed into clinical studies, only a single non-antibody scaffold, the Kunitz domain DX-88 (ecallantide; Dyax) has been granted regulatory approval (Box 1), with no further approvals since 2009. Notably, the development of several candidates, including DX-890 and AMG-220, has been halted in recent years

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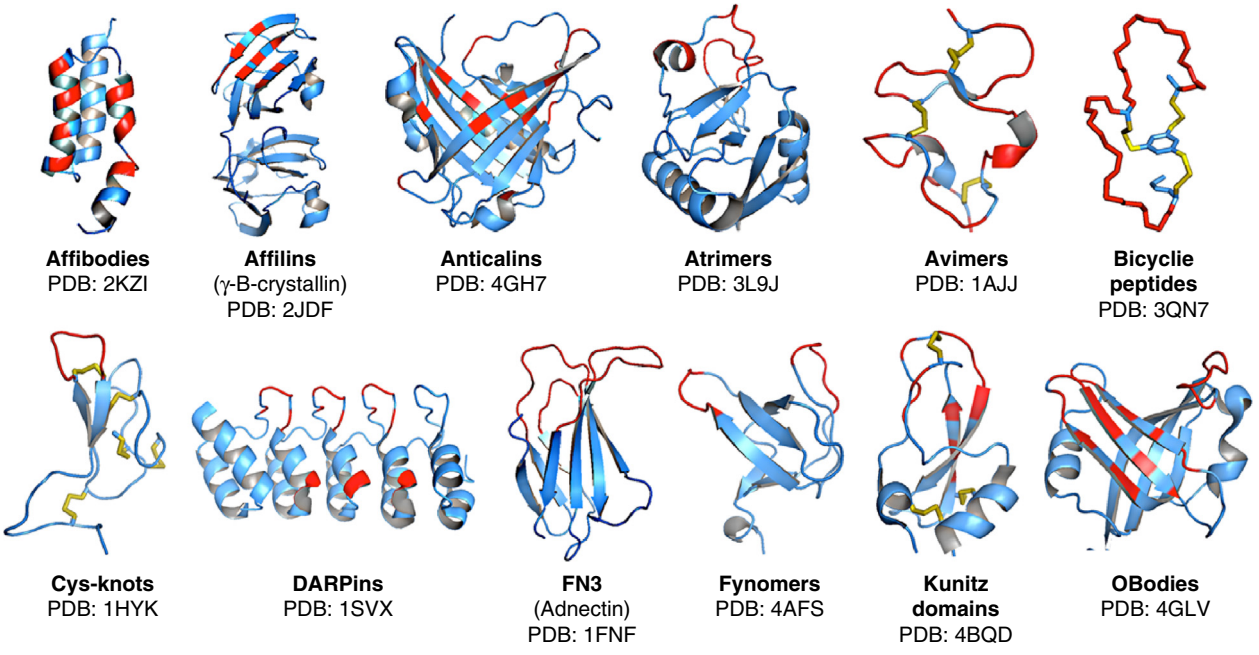

Scaffold	Parental protein	Structure	Randomization	MW (kDa)
Affibodies	Z domain (protein A)	α-helical	Helix randomization	6
Affilins	γ-B-crystallin	β-sheet	Beta-strand randomization	20
	Ubiquitin	α/β	Beta-strand randomization	10
Anticalins	Lipocalin	β-sheet + α-helical terminus	Loop randomization Beta-strand randomization	20
Atrimers	C-type lectin (tetranectin)	α/β	Loop randomization	3 × 20
Avimers	A-domain	Ca^{2+} binding Disulfide constrained	Loop randomization	4
Bicyclic peptides	Peptide	Chemically constrained	Loop randomization	2
Cys-knots	Peptide	β-sheet Disulfide constrained	Loop randomization	4
DARPins	Ankyrin repeats	α-helical + β-turn	Helix randomization Beta-turn randomization	14-21
FN3 scaffolds (Adnectins, Centyrins, Pronectins, Tn3)	Fibronectin (type III)	β-sheet	Loop randomization Beta-strand randomization	10
Fynomers	SH3 domain (fyn kinase)	β-sheet	Loop randomization	7
Kunitz domains	Serine protease inhibitor	α/β Disulfide constrained	Loop randomization	7
OBodies	OB-fold	β-sheet	Loop randomization Beta-strand randomization	12

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FIGURE 1

Structural features of non-antibody scaffolds. Non-antibody scaffolds under active development broadly fall into two classes, namely domain-sized compounds (Affibodies, Affilins, Anticalins, Atrimers, DARPins, FN3 scaffolds, Fynomers, Kunitz domains and OBodies) and constrained peptides (Avimers, Bicyclic peptides and Cys-knots). The former class averages around 6–20 kDa, with constrained peptides falling into a lower range of molecular weights at around 2–4 kDa. Illustrations were generated using PyMOL, positions diversified in the scaffolds are highlighted in red, disulfides are highlighted in yellow.

TABLE 1

Protein scaffolds under clinical and preclinical development

Scaffold	Company	Target protein	Drug name	Indications	Status
Adnectins [44,76–79]	Bristol-Myers Squibb	PCSK9	BMS-962476	Hypercholesterolemia	Phase I NCT01587365
		VEGFR2	Angiocept	Cancer	Phase II NCT00851045 NCT00850577 NCT00562419
		Myostatin	BMS-986089	Cachexia	Phase I NCT02145234
		EGFR/IGF-1R	n/a	Cancer	Preclinical
Affibodies [10,45,50,80–84]	Affibody	HER2	ABY-025	Cancer (PET imaging)	Phase I, II NCT01858116 NCT01216033 NCT02095210
		Complement protein C5	SOBI002	Inflammation	Phase I NCT02083666
		IL-17	ABY-035	Autoimmunity	Preclinical
		TNF/IL-6 (AffiMab)	n/a	Inflammation	Preclinical
		EGFR, IGF-1R, PDGFRβ, HER3, VEGFR2	n/a	Cancer	Preclinical
Affilins [85,86]	Scil Proteins	Fibronectin EDB splice variant	n/a	Cancer	Preclinical
		CTLA-4	PRS-010	Cancer	Preclinical
		VEGF-A	PRS-050 (Angiocal)	Cancer	Phase I NCT01141257
Anticalins [87–92]	Pieris	Hepcidin	PRS-080	Anaemia	Phase I NCT02340572
		IL-4Rα	PRS-060	Asthma	Preclinical
		HGFR	PRS-110	Cancer	Preclinical
		CD137/HER2	PRS-343	Cancer	Preclinical
		IL-23/IL-17	PRS-190	Autoimmunity	Preclinical
Atrimers [93–95]	Anaphore	IL-23	ATX 3105	Inflammation	Preclinical
		DR4	n/a	Cancer	
Avimers [49,96]	Avidia, Amgen	IL-6	AMG220	Crohn's disease	Phase I NCT00353756
Bicyclic peptides [97,98,100,101]	Bicycle Therapeutics	Kallikrein uPA HER2	n/a	Hereditary angioedema Cancer	Preclinical
Centyrins [102]	Janssen	HGFR	n/a	Cancer	Preclinical
		IL-17		Autoimmunity	
		TNF-α		Inflammation	
Cys-knots [103]	Medimmune	NaV1.7	n/a	Pain	Preclinical
DARPins [104–107]	Molecular Partners, Allergan	VEGF-A	MP0112 (Abicipar pegol)	Macular degeneration Macular edema	Phase II, Phase III NCT02462928 NCT02462486 NCT01397409 NCT02186119 NCT02181517
		VEGF-A/PDGF-B	MP0260	Macular degeneration	Preclinical
		VEGF/HGF	MP0250	Cancer	Phase I NCT02194426
		HER2	MP0274	Cancer	Preclinical
Fynomers [46,108]	Covagen	TNF/IL-17A (FynomAb)	COVA322	Plaque psoriasis	Phase I/II NCT02243787
		HER2 (FynomAb)	COVA208	Cancer	Preclinical
Kunitz domains [109–112]	Dyax	Kallikrein	DX-88 (Ecallantide)	Hereditary angioedema	Approved
		Neutrophil elastase	DX-890 (Depelstat)	Acute respiratory distress syndrome	Phase II NCT00455767
		Plasmin	DX-1000	Cancer	Preclinical
Obodies [113]	Obodies	n/a	n/a	n/a	Preclinical
Pronectins [114]	Protelica	VEGFR2, AXL tyrosine kinase, TNF-α, FZD receptors	n/a	Cancer, Autoimmunity	Preclinical
Tn3 [60,64]	Medimmune	CD40L	n/a	Autoimmunity	Preclinical
		TRAILR2		Cancer	

Abbreviations: CD40L, CD40 ligand; CTLA-4, cytotoxic T-lymphocyte antigen 4; DARPins, designed ankyrin repeat proteins; DR4, death receptor 4; ED-B, extra domain-B; EGFR, epidermal growth factor receptor; FZD, frizzled; HER2, human epidermal growth factor receptor type 2; HER3, human epidermal growth factor receptor type 3; HGFR, hepatocyte growth factor receptor; IGF-1R, insulin-like growth factor-1 receptor; IL-17A, interleukin-17A; IL-23, interleukin-23; IL-4Rα, interleukin-4 receptor alpha; IL-6, interleukin-6; PCSK9, proprotein convertase subtilisin kexin 9; PDGF-B, platelet-derived growth factor B; PDGFRβ, platelet-derived growth factor receptor β; PET, positron emission tomography; TNF, tumor necrosis factor; TRAILR2, TNF-related apoptosis-inducing ligand receptor 2; uPA, human urokinase-type plasminogen activator; VEGF-A, vascular endothelial growth factor A; VEGFR2, vascular endothelial growth factor receptor 2.

BOX 1

Clinical development of non-antibody scaffolds

Over 50 different protein scaffolds have been reported [6–8]. However, the majority of these scaffolds has exclusively been used in academic settings and only a subset is currently further developed commercially (Table 1). A single non-antibody scaffold has received approval by the FDA, the Kunitz domain Kalbitor® (ecallantide; Dyax), a plasma kallikrein inhibitor used for the treatment of hereditary angioedema [144,145]. Ecallantide was selected by phage display [146], inhibits kallikrein activity with picomolar affinity and displays a moderate half-life ($t_{1/2}$ ~2 h) in humans [140,145]. Phase III clinical trials demonstrated significant improvements of acute angioedema symptoms compared with placebo and a favorable safety profile, resulting in the approval of ecallantide in 2009 [147]. Competitors of the drug include the plasma-derived human C1-esterase inhibitors Berinert® (CSL-Behring) and Cinryze® (ViroPharma/Shire), as well as the bradykinin receptor antagonist Firazyr® (Shire). In addition to ecallantide the Kunitz domain DX-890 (depelstat; Dyax), a neutrophil elastase inhibitor, has been assessed in clinical trials of cystic fibrosis [148]. The high stability of this domain enables pulmonary delivery [149], with Phase IIa clinical studies in 2008 resulting in reduced levels of the proinflammatory cytokine interleukin (IL)-8 in patients [111]. No clinical updates of DX-890 development have been reported in recent years.

Affibodies have been developed for imaging applications and allow the visualization of metastases *in vivo* using positron emission tomography (PET) [121,150]. The phage display selected compounds display picomolar affinity against the human epidermal growth factor receptor (HER2)-neu tumor antigen [151]. Owing to their particularly low molecular weight (6 kDa), the compounds are characterized by rapid blood clearance and a favorable biodistribution for imaging applications, as indicated by increased tumor:organ accumulation ratio in comparison with the anti-HER2 IgG monoclonal trastuzumab [19]. A second generation Affibody molecule ABY-025 (Affibody) is currently being evaluated as an imaging agent in Phase I/II clinical trials [80]. More recently, an Affibody compound targeting the complement protein C5 commenced Phase I clinical evaluation for the treatment of inflammatory conditions (Table 1).

Adnectin scaffolds directed against the vascular endothelial growth factor receptor (VEGFR)2 tumor antigen [152] have been reported and are currently under clinical investigation. BMS-844203 (angiocept; Bristol-Myers-Squibb) was selected through mRNA display and binds its target with nanomolar affinity, effectively inhibiting VEGFR2 activation and angiogenesis [153]. CT-322 was formulated as a fusion to polyethyleneglycol (PEG), increasing hydrodynamic radius and half-life (~4 days), while reducing renal clearance [78,154]. Phase I dose escalation studies in humans revealed that the compound was well tolerated, with a maximum dose of 2 mg/kg. Several Phase II studies are currently ongoing, including treatment of recurring glioblastoma [78,155]. Another Adnectin-based compound, BMS-962476, directed against the cholesterol regulator PCSK9, is currently under Phase I investigation for the treatment of hypercholesterolemia [77,156]. Preliminary data from this trial revealed that treatment was well tolerated and displayed high efficacy, with free PCSK9 levels reduced by at least 90% in patients receiving a single 0.3 mg/kg dose [157]. The latest Adnectin to have entered clinical trials is BMS-986089, a myostatin inhibitor for cachexia and muscular dystrophy indications, currently undergoing a Phase I dose-escalation study (Table 1).

AMG220 (C326; Avidia/Amgen) is an Avimer targeting IL-6, a pleiotropic cytokine associated with inflammatory and autoimmune conditions [158]. Selected by phage display, AMG220 displays bi-specificity to its interleukin target, as well as binding to the Fc domain of IgG (resulting in reduced renal clearance and FcRn recycling; Fig. 3). The compound has subpicomolar affinity for IL-6 [49] and displays a moderate serum half-life (~30 h) [96]. Phase I clinical trials of AMG220 in Crohn's disease revealed dose-dependent reduction in serum C-reactive protein, an inflammation biomarker synthesized by hepatocytes in response to IL-6 [96]. Despite its apparent efficacy, Amgen has suspended the clinical development of the compound [114,159,160].

Anticalins are among the more actively developed non-antibody scaffolds, with a high number of lead compounds under preclinical development – directed against CTLA-4 [90], hepcidin [161], hepatocyte growth factor receptor (HGFR; MET) [89], IL-4Rα [162] and IL-23/IL-17 [163] (Table 1). PRS-050 (Angiocal®; Pieris), is an antiangiogenic Anticalin targeting VEGF-A currently undergoing Phase I clinical investigation. The compound was selected by phage display, and displays picomolar affinity to the VEGF-A molecule, as well as to splice variants [164]. To increase serum half-life (to ~6 days) PRS-050 was coupled to PEG, and evaluated in a dose-escalating Phase I clinical trial in patients with advanced solid tumors [126]. The compound was mostly tolerated up to a dose 10 mg/kg, with no formal maximum dose reached. Rare occurrences of grade 3 and 4 adverse effects were observed in this trial, however most effects were classified as mild-to-moderate. PRS-050 treatment resulted in effective VEGF-A neutralization, as indicated by the absence of free antigen, and the detection of VEGF-A–PRS-050 complexes [88,126].

The ankyrin repeat protein (DARPin) MP0112 (Molecular Partners/Allergan) targeting retinal angiogenic disorders driven by VEGF-A is currently being assessed in clinical studies. These disorders include age-related macular degeneration (wet AMD) and diabetic macular edema (Table 1). MP0112 was selected by ribosome display and displays picomolar affinity for its target [137]. Phase I studies of intravitreal injection into the human eye revealed an ocular half-life of ~13 days, with no detectable compound levels in serum. A single treatment dose resulted in undetectable levels of ocular VEGF-A for up to 4 weeks in all patients. Preliminary efficacy data obtained from a limited number of patients indicated reduced edema and improved visual acuity [137]. A subsequent Phase I/II study supported these observations and parallel studies on wet AMD showed favorable outcomes for this indication, including a dose-dependent reduction of vascular leakage [106,165]. MP0112 (abicipar pegol) entered Phase III clinical trials in 2015 (Table 1). In addition to MP0112, the bi-specific DARPin MP0250 targeting VEGF/HGF has recently commenced clinical evaluation for the treatment of solid tumors [166].

(Box 1). Although the reason for discontinuation of each specific candidate varies, it is evident that, as scaffolds have evolved from innovative binding moieties to potential drug candidates, additional requirements have emerged. For example, one such requirement is the ability to match, and preferably exceed, potencies that can be achieved by monoclonal antibodies. The quest for differentiation faces three major challenges, namely serum-half life, tissue penetration and advances in monoclonal antibody technology.

Serum half-life

Repeated passages in serum significantly increase the uptake of therapeutic proteins into tissue and organs. This is exemplified by human IgG monoclonals, which display long serum half-lives (up

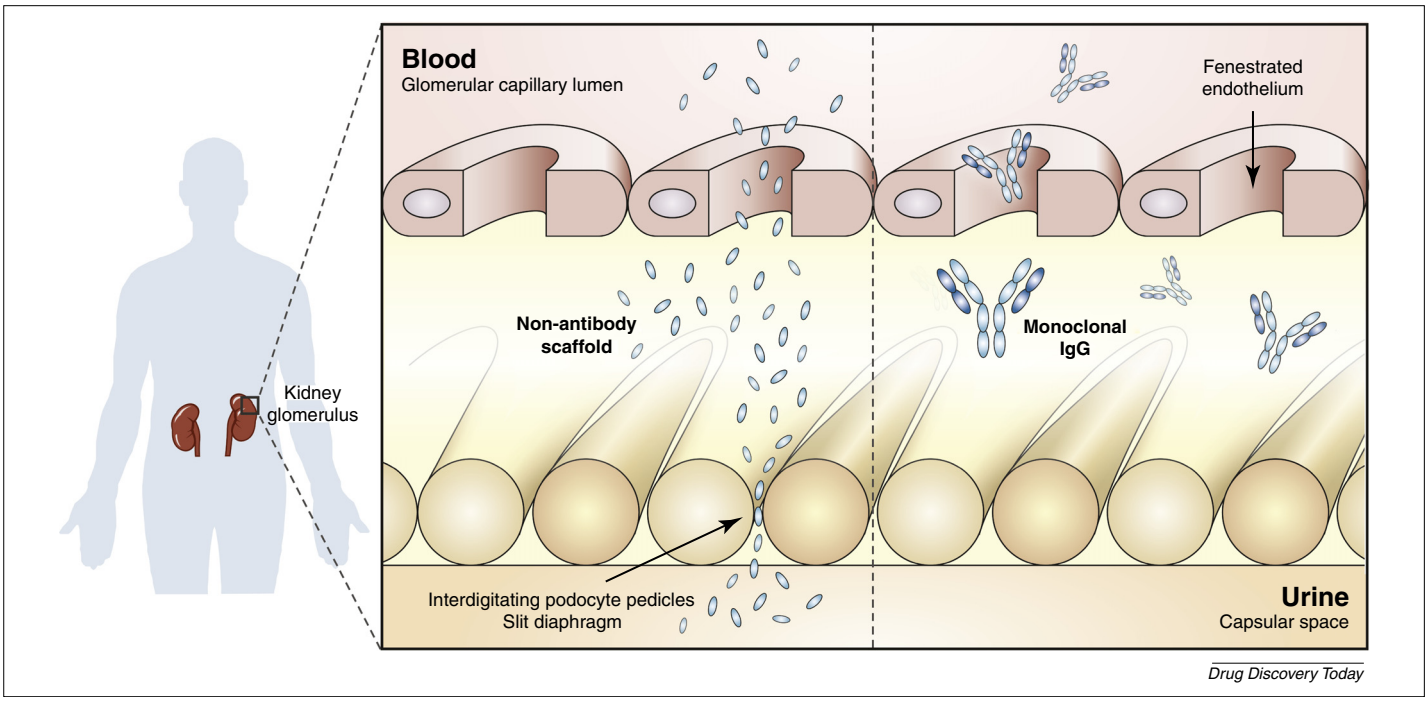

FIGURE 2

Serum half-life of scaffolds and monoclonal antibodies. Non-antibody scaffolds are rapidly removed from the bloodstream, through filtration by the slit diaphragm of interdigitating podocyte pedicles. Monoclonal antibodies of the IgG isotype do not underlie renal filtration, as a result of their molecular weight of ~150 kDa, well above the renal filtration barrier (~60 kDa).

to several weeks) [9]. By contrast, unmodified non-antibody scaffolds are largely eliminated through the kidney after a single pass (Fig. 2) [10]. The lack of intrinsic half-life extension immediately puts scaffolds at a disadvantage, by limiting their ability to accumulate at a site of pharmacological action. Consequently, most scaffold-based drug candidates require the use of protein engineering or formulation to ensure continuous exposure after dosing.

Protein engineering can be used in multiple ways to extend the circulating half-life of therapeutic proteins. The first approach is based on increasing size to minimize single-pass kidney filtration. This requires an increase above a critical mass of or above ~60 kDa (renal filtration cut-off) and is generally achieved through genetic fusion with a larger protein or alternatively through conjugation with an organic polymer, such as polyethylene glycol (PEG). The first approach involves genetic fusions with the Fc region of human IgG, or alternatively with serum albumin (both of which interact with FcRn). This strategy is based on exploiting the salvage mechanism mediated by the neonatal Fc receptor (FcRn) expressed on vascular endothelial cells (Fig. 3) [11]. FcRn prevents lysosomal degradation, and recycling can be achieved through direct and indirect strategies [12]. Indirect mechanisms that trigger salvage through FcRn include the engineering of scaffolds to bind IgG or albumin (rather than a direct genetic fusion to these molecules). This approach requires binding to these two serum proteins in ways that do not interfere with their respective FcRn interactions; it could however allow the generation of overall smaller constructs than genetic fusions to antibody Fc regions.

Many examples of scaffold half-life extension have been reported, see [13] for a review. However, it is important to note that the FcRn pathway displays limited efficacy for molecules with a molecular mass under the renal filtration cut-off, because clearance after a single pass through the kidney tends to be the more dominant mechanism [14]. Therefore, unless advanced formulation or delivery technologies are used, the minimal size for a parenterally administered and infrequently dosed biopharmaceutical drug effectively is ~60 kDa. Unfortunately, the very addition of a 'half-life solution' could thereby limit or even eliminate one of the key advantages of non-antibody scaffolds, namely their relatively small size and low molecular weight (2–20 kDa). An increase in mass could also reduce other advantageous properties of these molecules, such as high soluble expression and stability (Box 2). Scaffold-based molecules with long plasma half-lives thereby risk resembling IgG-like molecules with overall limited differentiation.

Tissue penetration

It is well documented that a low-molecular-weight therapeutic, if of sufficiently high affinity to the target, will distribute more effectively into tissue (Fig. 3), and extensive clinical data, particularly relating to drug penetration into solid tumors, has been reported [15,16]. This is particularly the case for smaller scaffolds in the 2–4 kDa range, which are expected to extravasate and diffuse into tissue effectively [17]. Whereas tissue penetration is a recognized and crucial factor in the design of novel oncology treatments, it is also relevant for other indications, if significant organ diffusion is required following parenteral administration. Examples include targeting less-vascularized tissues and organs, such as intestine, adipose tissue and muscle, as well as renal and lung lumina where, in each case, antibody IgG penetration is limited [18]. Owing to their relatively low molecular weight, unmodified scaffolds (e.g. without half-life extension) display relatively fast tissue penetration in comparison with human IgG

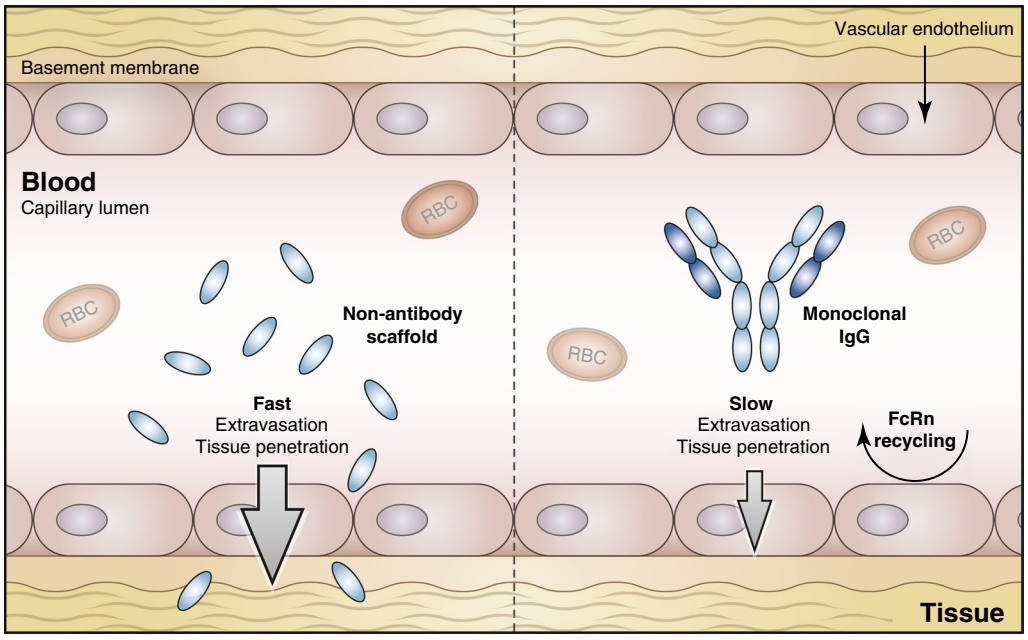

FIGURE 3

Tissue penetration of scaffolds and monoclonal antibodies. Non-antibody scaffolds are capable of high levels of tissue penetration, owing to fast extravasation from the capillary lumen through the vascular endothelium and basement membrane. By contrast, monoclonal antibodies of the IgG isotype are characterized by slow tissue penetration. Unlike unmodified scaffolds, monoclonal antibodies display long serum half-lives, as a result of reduced renal filtration and recycling by FcRn present in the vascular endothelium [11]. *Abbreviations*: RBC, red blood cell; FcRn, neonatal Fc receptor.

monoclonals (Fig. 3) [19–21]. In combination with short serum half-lives, this can be exploited to engineer compounds with excellent signal:noise ratios in imaging applications (Box 3) [22]. A successful example is the development of radiolabeled Affibody molecules for the imaging of human epidermal growth factor (HER2)-positive tumors [19].

However, for therapeutic applications, a general advantage of non-antibody scaffolds is less apparent. Owing to their large molecular weight of ~150 kDa, human IgG antibodies were initially considered to be limited to targets accessible directly from the vasculature [7]. However, more-recent clinical data showed that if dosed highly enough monoclonal antibodies can distribute effectively in less-vascularized tissue [18]. The limiting factor of antibody therapy in respect to tissue penetration therefore does not just relate to the molecular mass of the therapeutic molecule but also to the therapeutic index (i.e. dose at which toxicities will occur) and cost of manufacture (i.e. total dose per patient per year). As long as dose-limiting toxicities occur at doses that are significantly higher than those required for efficient tissue penetration (typically 1–10 mg/kg for IgG), antibody therapeutics can effectively access most tissues in the body. Indeed, owing to their intrinsically high potency, selectivity and specificity, therapeutic indexes of antibodies are often large, enabling high doses to be used clinically that are safe and well tolerated. Notable exceptions relate to the brain and central nervous system [23,24]. In these areas, although high doses will still lead to relatively higher uptake, total drug levels are likely to be too low for significant pharmacological action [25].

In summary, the need for alternative, highly tissue penetrating scaffolds only arises if toxicities occur at doses insufficient for effective IgG tissue penetration (as can be observed after conjugation with cytotoxic agents, see section below) or if the antibody dose required increases costs of goods to a commercially unviable level. It should also be noted that the total dose is influenced by the amount of drug required for the desired pharmacology and the frequency of administration. For instance, an unmodified scaffold antagonist might be administered at one-tenth of the dose of an equivalent IgG therapeutic, but will require daily instead of bi-weekly or monthly dosing. Scaffolds, however, could offer advantages for locally administered therapeutics (such as for intravitreal injection for ocular diseases), owing to their lower molecular weight and higher solubility, and for applications in which Fc-mediated immune effector functions are unnecessary or undesirable (for instance the neutralization of soluble signaling factors).

Advances in monoclonal antibody technology

Human antibodies of the IgG isotype are characterized by long serum half-lives in the range of days to weeks [9]. Antibody half-life can be further increased to timescales of several months, provided that there is low target-mediated clearance, by engineering the interaction of the antibody Fc region with the FcRn receptor, resulting in increased uptake and favorable dosing regimens [26]. Protein engineering approaches have also allowed the generation of smaller human antibody reagents, thereby reducing the size-advantage of non-antibody scaffolds. For instance, Fcabs (F-star) eliminate the requirement for antibody variable domains, and instead rely exclusively on the antibody Fc region, by engineering novel antigen-binding sites into constant regions [27]. These antibody variants combine the size advantage of a scaffold with the intrinsic antibody half-life extension mechanism

BOX 2

Differentiation among non-antibody scaffolds

The large number of non-antibody scaffolds under development, encompassing a wide range of protein architectures, raises the question of differentiation, and whether certain scaffold types might be particularly suited to specific applications. In terms of architecture and molecular weight, scaffolds fall into two classes (domain-sized and peptide-derived; at 6–20 kDa and 2–4 kDa, respectively). This difference in molecular weight directly relates to differences in production, with peptide-related compounds having the additional advantage of being amenable to manufacture by chemical synthesis [167,100]. By contrast, domain-sized scaffolds generally need to be expressed in bacteria, or alternatively in CHO-, *Pichia-pastoris*- or HEK-293-based eukaryotic systems (Table I). Overall lower molecular weight compared with IgG therapeutics is also considered to be of particular importance for imaging applications (Box 3) and could allow the targeting of otherwise cryptic epitopes within protein clefts and other poorly accessible structural features [100,168,169].

When conducting comparative analyses it should be noted that many scaffolds are proprietary to biopharmaceutical companies and that data access might be restricted. Moreover, published studies could well have been conducted using varying conditions and methodologies, further complicating analyses. Nevertheless, it is evident that there appear to be more similarities than differences between the properties of different scaffold types. This is perhaps not surprising considering similar driving forces faced by companies developing non-antibody scaffolds as human therapeutics. For example, most of the parental scaffold proteins have relatively high melting transitions, such as a T_m of >80 °C for Adnectins [170], 79 °C for Anticalins [171] and ~70 °C for Fynomers [108] – up to ~120 °C for the recently developed Alphabodies [172] – and most are reported to be well expressed in bacteria. By contrast, relatively large variation of properties can be observed among binders of a single scaffold type (Table I). For instance, large differences in melting temperatures have been reported, depending on the actual sequence and antigen specificity of each selected binder. This behavior is probably caused by the small size of the scaffolds, resulting in the effect that relatively minor sequence changes can induce profound changes of overall biophysical or pharmacokinetic properties [173]. Owing to their smaller size (2–20 kDa), residues in non-antibody scaffolds could be involved in high affinity binding, as well as important biophysical properties, such as thermodynamic or colloidal stability [174]. Recognition of different molecular targets could therefore have direct impact on a wide range of scaffold characteristics, which is in marked contrast to human antibody therapeutics of the IgG isotype (150 kDa). Although the effect of sequence variation could in principle be analyzed by computational or high-throughput methods, further releases of preclinical and clinical data will ultimately be necessary to evaluate robustness and applicability of different scaffold types.

TABLE I

Biophysical and pharmacokinetic properties of non-antibody scaffolds.

Scaffold	Expression	Stability	Half-life (*in vivo*)
Adnectins	*Escherichia coli* 20–40 mg/l [115]	T_m parent = 84 °C [116] T_m selected = 37–73 °C [115,117]	>53 h (PEG) [78]
Affibodies	Peptide synthesis [118] *E. coli* [119]	T_m parent = ~75 °C [120] T_m = 42–71 °C [118]	4–14 min [121]
Affilins	*E. coli* 100 mg/l [85]	γ-B-crystallin-based T_m parent = 80 °C T_m selected = 56–72 °C [85] Ubiquitin-based T_m parent = 82 °C T_m selected = 54–66 °C [122]	n/a 20–56 h in mice (PEGylated, Fc- or MSA-fused) [122]
Anticalins	*E. coli* 2–20 mg/l [123]	T_m parent = 79 °C T_m selected = 53–73 °C [123–125]	~6 d (PEG) [126]
Atrimers	*E. coli* [127]	Stable at 70–80 °C [95]	~24 h (parental tetranectin)[95]
Avimers	*E. coli* ≥ 1.4 g/l [49]	No aggregation after 2 weeks at 50 °C [49]	30 h (Fc binding domain)[96]
Bicyclic peptides	Peptide synthesis [100] HEK-293 ≥80 mg/l [128]	n/a	30 min in mice [129] >24 h in mice (ABP- or Fc-fused) [128,129]
Centyrins	*E. coli*	T_m parent = 93 °C T_m selected = 46–87 °C [102]	n/a
Cys-knots	*E. coli* 5–20 mg/l [130,131] Peptide synthesis [132]	T_m > 100 °C [133,134]	<35 min in mice [135]
DARPins	*E. coli* Up to 200 mg/l [136]	T_m > 66–89 °C [35,136]	≥ 13 d (PEG, ocular half-life) [137] 3 min in mice [20]
Fynomers	*E. coli* 24–78 mg/l [138] CHO 3.5–20.7 mg/l [46,139]	T_m parent = ~70 °C [139]	68 h in mice (Fc-fused) [139]
Kunitz domains	*Pichia pastoris* [140]	≥75% active after heating to 95 °C [140,141]	2 h [142]
OBodies	*E. coli* 100–200 mg/l [113]	T_m = 66–81 °C [113]	n/a
Tn3	*E. coli* 40–400 mg/l [60]	T_m = ~83 °C [143]	36 min in mice [60]

Abbreviations: ABP, albumin-binding peptide; CHO, Chinese hamster ovary cells; Fc, fragment crystallizable; HEK, human embryonic kidney cells; MSA, mouse serum albumin; PEG, polyethylene glycol; T_m: melting temperature.

BOX 3

Imaging applications of non-antibody scaffolds

Non-antibody protein scaffolds (including Affibodies, Bicyclic peptides, Cys-knots, DARPins, Anticalins and Adnectins) have been successfully conjugated to radionuclides and near-infrared (NIR) fluorescent dyes for *in vivo* imaging of solid tumors, reviewed in [175]. These reagents with affinities down to the nanomolar to picomolar range have several biophysical properties that make them ideal candidates for molecular imaging in preclinical and clinical studies. Important properties that distinguish them from antibody-based reagents are the absence of the immunoglobulin Fc region and an overall small size (2–20 kDa), enabling efficient capillary extravasation and tissue diffusion (Fig. 3). This behavior is particularly relevant to diagnostic applications in oncology [176,177]. In addition, unmodified scaffolds that do not include antibody Fc regions or bind to IgG or albumin, display short serum half-life and are rapidly cleared from the body (Fig. 2). Such protein scaffolds offer the potential of increased signal:noise ratios in comparison to antibody-based imaging reagents. This is exemplified by the iodinated ABY-002 Affibody molecule, which displays excellent bioavailability and image contrast when benchmarked against the anti-HER2 monoclonal antibody trastuzumab (Herceptin®; Genentech) [19].

More recently, multiple studies have provided insights into the biophysical characteristics that maximize tumor uptake and minimize off-target effects. In addition to low molecular weight as a key determinant of vascular permeability and tissue accessibility [177,178], these studies have also revealed other effects that influence the retention of the label in the tumor. These include affinity and avidity of the scaffold, as demonstrated for DARPins [20] and Affibodies [151], and whether it is internalized and degraded [179]. In addition, overall hydrophilicity has been shown to determine whether the scaffold is retained in off-target sites such as the liver or the kidneys. This has been demonstrated for Adnectins, which are relatively hydrophilic and have been shown to have longer renal than hepatic retention [180]. Similarly, removal of electric charges from Cys-knots [181] and Adnectins [180] has been shown to reduce renal retention. Importantly, chelators used to conjugate protein scaffolds to radionuclides for positron emission tomography (PET), single-photon emission computed tomography (SPECT) and scintigraphic imaging, and NIR dyes for optical imaging have the potential to alter size, affinity and charge. For example, the substitution of a positively charged lysine residue with the neutral serine has been shown to decrease renal retention of an Affibody molecule [182].

An innovative concept, originally developed to improve the delivery of therapeutic payloads and imaging reagents by monoclonal antibodies, is the use of pre-targeting with a bi-specific molecule [183]. The approach involves the use of bi-specifics recognizing tumor antigen and a small ligand, which can be administered at a later stage after the antibody has been cleared from the circulation and from off-target sites. This ligand can serve as a vehicle for payload delivery for therapeutic or imaging applications. Examples include an Anticalin domain specific for fluorescein fused to an antibody specific for a tumor antigen. This pre-targeting strategy has enabled sensitive *in vivo* imaging of tumors using a fluorescein–NIR-dye conjugate [184].

through FcRn binding and could potentially offer significant tumor uptake per injected dose by combining the advantages of small size and long circulating half-life [27,28]. The clinical evaluation of an anti-HER2 Fcab™ (NCT02286219) will determine whether these favorable pharmacokinetic properties are translatable into humans.

Although non-antibody scaffolds often display high thermodynamic stabilities, it is evident that the stability of human antibody reagents can be increased through well-established protein engineering approaches [3]. A generally low propensity to aggregate is another desirable property of non-antibody scaffolds; however, methods for increasing the aggregation resistance of human antibody reagents are increasingly becoming available [3,29–31]. Finally, the cost of production of non-antibody scaffolds is potentially lower, because of the possibility of using bacterial rather than mammalian expression systems [32,33]. However, with a large number of antibody therapeutics transitioned into commercial production and increased economies of scale, the cost of goods for CHO-cell-expressed antibody IgG therapeutics is decreasing, and can often be adequately controlled through efficient chemistry, manufacturing and control (CMC) processes [32].

Strategies for the generation of differentiated non-antibody scaffold drugs

To overcome challenges associated with serum half-life, tissue penetration and advances in monoclonal antibody technology, a wide range of protein engineering and drug delivery approaches have been applied to non-antibody scaffolds. Here, we discuss current advances and propose further areas of innovation.

Formulation and novel delivery devices

Owing to the problems associated with half-life extension the use of slow-release formulations and innovative drug delivery, devices could become an attractive strategy for non-antibody scaffolds. Provided the required efficacious total dose is sufficiently low (in terms of mg), both approaches can enable high serum concentration, despite the use of drug candidates with intrinsically short serum half-life. Slow release formulation has a long track record in the administration of agonistic peptides (including Zoladex® and Bydureon®), but has not been extensively applied to non-antibody scaffolds [34]. This is because of the antagonistic pharmacology of most scaffold-based drug candidates, which generally require relatively high doses to neutralize a significant proportion of the target molecule stoichiometrically. Such doses had been considered too high to be formulated successfully through a slow-release approach. However, with recent reports of scaffolds engineered to reach antibody-like affinities in the picomolar [35] to femtomolar [36] range, such limitations might no longer apply for next-generation candidates. With the typical molecular weight of a non-antibody scaffold at one-tenth of an IgG monoclonal (assuming unmodified scaffolds), doses of up to 10 mg per administration could thereby become feasible, which are accessible through slow-release formulation. Alternatively, innovative drug delivery devices, such as mini-pumps, are increasingly becoming available. Such devices were pioneered in the treatment of diabetes patients, and are currently in late-stage clinical testing [37]. Examples include exenatide, a short-half-life glucagon-like peptide (GLP-1) receptor agonist, administered through up to yearly dosing. Significant opportunities exist through the use of such devices

to turn potent but short half-life scaffolds into differentiated drug candidates. This area has remained largely unexplored, however it might well see increasing interest as companies try to find new ways to generate candidates out of their often significant investment in scaffold technologies [38,39].

Multi-specificity

The concept of dual (or multiple) specificity has attracted considerable attention in recent years [40–42]. Many approaches focus on the concept of taking two or more validated biologics and combining them in one construct. Such constructs have the ability to target either multiple proteins or multiple epitopes on the same molecule simultaneously [43] and several examples of scaffold-based constructs have been reported (including DARPins, Anticalins, Affibodies, Adnectins and Fynomers) [44–50]. The latter strategy can potentially lead to enhanced neutralization efficacy [51] or to increased clearance through the induction of large immune complexes [52]. Although considerable advances have been made in the design and manufacture of antibody-based bi- or multi-specifics, challenges remain. In particular, accurate prediction of the resulting combined pharmacology and the accurate adaptation of each pharmacological activity to result in a potent but nontoxic molecule at, effectively, a fixed dose have turned out to be more complex than first anticipated. Furthermore, a multi-selective biologic might require multiple biomarkers to assess the therapeutic window accurately for each pharmacological moiety, resulting in a significant increase in development costs – discussed in further detail, in particular in comparison to polyclonal antibody approaches, in a recent review on bi-specifics [53]. Consequently, a majority of the limited number of non-cell-crosslinking, bi-specific antibodies progressing to pivotal Phase IIb studies target highly related biological pathways, thereby simplifying the translational medicine and safety strategy. Examples include antibodies against IL-4/IL-13 (Sanofi) [54], Her1/Her3 (Genentech/Roche) [55] or Her2/Her3 (Merrimack Pharmaceuticals) [56]. However, the targeting of related pathways could also ultimately limit the differentiation of the resulting bi-specific versus the mono-specific parental antibodies or, indeed, combinations of the parental antibodies.

The remaining technical complexities of generating multi-specific antibody therapeutics open up possibilities for the development of non-antibody scaffolds, either on their own or as an 'add-on' to antibody therapeutics [51,50]. Indeed, multimerization plays to the advantage of scaffold platforms, which provide highly soluble, stable and well-expressing monomer building blocks for such strategies. Moreover, a potentially unique feature of scaffold multimerization is the ability to generate potent agonists, which can be readily achieved for targets that can be activated through simple di- or tri-merisation. Examples for this behavior include the tumor necrosis factor (TNF) family of receptors [57] and many G-protein-coupled receptors (GPCRs) [58]. The approach is exemplified by the targeting of TRAIL-R2 (also known as death receptor 5, DR5) with several candidates under preclinical [59,60] and clinical [61] development. Multimerization has also been described as a pragmatic way to boost the potency of the often more weakly binding monomer [62,63]. Scaffold multimerization is not without challenges, for instance repeating identical epitopes within a molecule can trigger innate immunity [64]. Protein multimerization can also initiate aggregation processes, which increase the immunogenic potential (Box 4) [64,65]. However, the high intrinsic stability of non-antibody scaffolds is likely to limit such processes [7].

BOX 4

Immunogenicity of non-antibody scaffolds

Immune responses to administered protein therapeutics remain a significant challenge for clinical success. For non-antibody scaffolds immunogenicity can represent a greater challenge than for antibodies, particularly for those derived from non-human or synthetic proteins. An innovative approach relates to the recent development of scaffolds chemically synthesized with D-amino acids (Reflexion Pharmaceuticals) [185]. These compounds are mirror images of their parental protein G B1-domains and are expected to be structurally nonimmunogenic [186].

To date, there have been few reported clinical trials of non-antibody scaffolds. Kalbitor® (ecallantide; Dyax), currently the only non-antibody scaffold protein therapeutic approved by the FDA, has undergone extensive clinical trials for the treatment of hereditary angioedema [187,188]. In 216 patients treated with ecallantide, 36 (17%) developed anti-drug antibodies, along with several reported cases of patients developing antibodies to *Pichia pastoris*, the organism used to manufacture the drug. BMS-844203 (angiocept; Bristol-Myers-Squibb), an Adnectin targeting vascular endothelial growth factor receptor (VEGFR)2 has been assessed at escalating intravenous doses in patients with advanced solid tumors [78]. This study reported anti-drug antibodies in 31 of 38 (82%) patients. Subsequent epitopic analysis revealed that the anti-drug antibodies bound to the engineered binding loops of the Adnectin. A Phase I study of PRS-050 (Angiocal®; Pieris), an Anticalin targeting VEGF-A, in 25 patients with advanced solid tumors also reported no anti-drug antibody responses in any of the patients [126].

It remains difficult to draw general conclusions from what is so far a small number of clinical studies, in terms of predicting immunogenicity and the effects on safety and efficacy. Moreover, immunogenicity is a complex phenomenon that is known to be influenced by factors outside of the molecular structure of the therapeutic (including dosing, formulation, excipients, the presence of contaminants such as host cell proteins or degradation products, the route of administration, and the immune and genetic status of the patient) [189]. Although preclinical assessment of immunogenicity risk can be conducted using *in silico* or *in vitro* prediction methods or by *in vivo* assessment of anti-drug responses in rodents or non-human primates [190], clinical data required to determine relative utility in predicting anti-drug responses in patients is not yet publically available. Further clinical studies of non-antibody scaffold therapeutics will ultimately be required to address the relative immunogenicity of different scaffolds classes.

Drug conjugates

Although nonmodified IgG antibodies typically show toxicities only at super-pharmacological doses, they are more common for antibody–drug conjugates (ADCs) at even moderate dose levels [66]. These toxicities can be dose-limiting in the clinic, and in turn limit the penetration of the ADC into solid tumors (this is in contrast to liquid tumors where malignant cells are mostly accessible in serum and generally less affected by dose restrictions) [59]. Scaffolds have the potential to offer significant advantages in such a scenario, by delivering a toxic moiety deep into a solid tumor mass while being rapidly cleared from systemic circulation. Early

ADC designs were based on human antibody fragments. Examples include moxetumomab pasudotox (Medimmune/AstraZeneca), a CD22-targeting antibody fragment fused to a shortened *Pseudomonas* exotoxin, which has now reached Phase III trials [67]. Furthermore, antibody-based ADCs can suffer from aggregation owing to the hydrophobicity of the attached chemical payload [68,69]. Stable and nonaggregating scaffolds can therefore provide an attractive alternative in such situations, in particular if an overall small molecular weight compatible with high tumor uptake can be maintained [17,70] and a wider range of conjugation chemistries can be explored compared to working with IgG antibodies [71]. A number of reports demonstrate that scaffolds, in particular constrained peptides, unmodified and as conjugates to radionuclides, are highly metabolically stable [72,73] and were shown to be secreted intact in the urine [74]. This high metabolic stability is achieved by working with fully synthetic molecules where a stable and highly inert linkage between the scaffold peptide and the conjugate can be easily selected.

Concluding remarks

Over the past decade, a large number of pharmaceutical companies have invested significantly in either acquiring or building in-house scaffold technologies. The first generation of such non-antibody drug candidates is now progressing into clinical trials. However, challenges remain, and the wider translation of scaffold drugs into clinical practice will require additional innovation. Although a handful of promising and differentiated scaffold drug candidates continue to advance clinically, it could be their use as drug conjugates, or the combination with advanced formulation and delivery technology, that will ultimately drive value creation. Considering that the expenditure of developing over 50 scaffold technologies will have to be recovered, this will require considerable time and the development of new and differentiated therapeutic leads. Scaffold-based platforms could also allow the development of fast and efficient diagnostics, resulting in a reduced cost of clinical imaging, an application with significant promise. Disruptive innovation could also result from further reducing the molecular size of the scaffolds down to the proportions of macrocycle drugs [75]. This could open up new and exciting opportunities, including the targeting of intracellular interactions and oral administration. In summary, further creativity will be required to transform this promising class of binding molecules into validated therapeutic and diagnostic modalities.

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