Trends in
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Review

Biomolecules capturing live bacteria from clinical samples

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Rapid phenotypic antimicrobial susceptibility testing (AST) requires the enrichment of live bacteria from patient samples, which is particularly challenging in the context of life-threatening bloodstream infections (BSIs) due to low bacterial titers. Over two decades, an extensive array of pathogen-specific biomolecules has been identified to capture live bacteria. The prevailing biomolecules are immune proteins of the complement system, antibodies, aptamers, phage proteins, and antimicrobial peptides. These biomolecules differ by their binder generation technologies and exhibit highly variable specificities, ranging from bacterial strains to most pathogenic bacteria. Here, we summarize how these diverse biomolecules were identified, list examples of successfully reported capture assays, and provide an outlook on the use of nanobodies raised against conserved surface-accessible proteins as promising biomolecules for pathogen capture.

Highlights

The antimicrobial resistance (AMR) crisis represents one of the greatest medical challenges for global health.

Rapid diagnostics is key to slow down the spread of AMR.

Biomolecules that enable the capture of live bacteria are an essential cornerstone to overcome growth-dependent time bottlenecks and to accelerate phenotypic diagnostic tests.

A large number of biomolecules are suitable for live bacterial capture, but a predominant class of such capture agents has not yet emerged.

NestLink is a recently developed robust technology platform that enables the generation of nanobodies against conserved outer membrane proteins of Gram-negative pathogens.

Rapid diagnostics of bloodstream infections is critical for patient safety and antimicrobial stewardship

BSIs (see Glossary) are routinely diagnosed by blood culture, and results are typically obtained within 1 or 2 days and occasionally only within 5 days [1]. Blood culturing requires several time-consuming steps, including selective culture enrichment, differential plating, biochemical/serological testing, and finally, **AST**. Delayed sepsis diagnostics lead to unnecessary use of broad-spectrum empirical antibiotic therapies and longer durations of hospital stays [1]. Additionally, inappropriate antibiotic therapy at the onset of a BSI has been associated with higher mortality [2]. Therefore, broad coverage by the empiric antibiotic and rapid bacterial species identification is crucial for patient outcome, especially in patients who are critically ill [3]. However, broad-spectrum antibiotics are potent drivers of **antimicrobial resistance (AMR)** [4], which is harmful to the individual patient and causes serious health problems at the level of the hospital and the community as a whole. Indeed, AMR is considered by WHO as one of the greatest public health threats [5,6].

Early diagnosis of pathogens and their associated AMR patterns would enable fast de-escalation of the empirical broad-spectrum antibiotic, thereby adjusting the treatment to a pathogen-specific and, thus, adequate antibiotic therapy. To increase the lifespan of the existing arsenal of antibiotics and slow the spread of AMR, WHO has called for rapid diagnostic tools to optimize adequate use of antibiotics [6]. Rapid molecular techniques carried out in blood cultures for diagnosis of BSIs have already been shown to be cost-effective and reduce the time for effective treatment, length of stay, and mortality [7]. In addition, the positive effect of rapid BSI diagnostics was found to be increased when the program was accompanied by an **antimicrobial stewardship** program [8].

Despite remarkable progress in molecular techniques, the **sensitivity** of BSI diagnostics remains disappointingly low. The most promising recent advance in molecular platforms for sepsis

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diagnosis relies on PCR-based methods. However, they differ in the detection of the amplicons, that is, molecular probes (SeptiFast), electrospray ionization mass spectrometry (Iridica), or T2 magnetic resonance technology (T2 bacteria) [9]. The SeptiFast assay from Roche Molecular Diagnostics, one of the first molecular platforms, has a sensitivity of 68–75% and a **specificity** of 86–92% depending on studies [10]. This might be due to high **limits of detections (LODs)**, the limited numbers of species that can be identified by multiplex PCRs, detection of dead or irrelevant pathogens (high rates of contamination), and a problematic standardization and interpretation of results [10]. As such, classical blood culture has not been replaced and remains the gold standard because of these technical limitations and the high costs of molecular diagnostic techniques. However, blood culture has its sensitivity limitations because up to 50% of blood cultures remain negative despite suspicious BSI symptoms [11]. In praxis, sepsis diagnosis often relies on inflammatory biomarkers, the detection of which is much faster than with blood culture [1], but unfortunately cannot distinguish infectious from non-infectious origin.

In an ideal world, BSI diagnostics would be rapid, sensitive, and specific, differentiate live versus dead pathogens, and allow for phenotypic antibiotic resistance testing. However, to achieve a faster time to results, the time-consuming blood culture step has to be replaced by an efficient capture step directly from patient blood without killing the captured bacterium. The major limitation to capturing intact live bacteria is sensitivity. Patients with sepsis have been reported to have pathogen loads of less than one colony-forming unit (CFU) per ml of whole blood [11]. At the same time, quantification of bacterial DNA by PCR methods indicates the presence of ~1000 genome copies in the same blood volume. Furthermore, bacterial cells are massively outnumbered by blood cells (mainly erythrocytes), which explains why BSI diagnostics is so challenging and time-consuming.

Many of the novel methods to enrich bacteria from blood have been developed for DNA-based diagnostic methods and, therefore, involve processes that kill bacteria [12]. As the main limitation of such enrichment procedures, the captured bacteria can no longer be analyzed by phenotypic AST, which is needed to determine an accurate and complete AST profile. In this review, we focus on biomolecules that allow for the enrichment of live pathogenic bacteria directly from patient samples, in particular from blood.

Biomolecules of the innate immune system broadly recognize pathogens

As a defense strategy, numerous molecules of the human innate immune system have evolved to detect a range of pathogens via recognition of common motifs called pathogen-associated molecular patterns (PAMPs). Some of these molecules, called pattern-recognition receptors (PRRs), caught the attention of the diagnostic field because of their capacity to recognize bacterial surface PAMPs shared by many different species. These are mostly cell wall peptidoglycans or bacterial carbohydrates, such as lipopolysaccharide (LPS) of Gram-negative bacteria or lipoteichoic acids (LTAs) of Gram-positive bacteria.

Fc-mannose-binding lectin

The engineered chimeric human opsonin protein Fc-mannose-binding lectin (FcMBL) is one of the most advanced biomolecules used to bind and capture a range of pathogens. FcMBL comprises the human MBL carbohydrate recognition domain fused to the Fc region of human IgG [13] (Figure 1). Native MBL is an essential component of the innate immune system and acts as an opsonin to activate the complement system via the lectin pathway [14]. It binds carbohydrate PAMPs from a range of pathogens, including bacteria, viruses, fungi, parasites, and toxins. Engineered FcMBL coupled with magnetic beads was shown to capture *Candida albicans*;

Glossary

Antimicrobial resistance (AMR): ability of microorganisms to withstand antibiotic treatment. Resistance mechanisms are genetically encoded, this being the underlying reason why AMR spreads as a consequence of antibiotic usage and its associated selection pressure.
Antimicrobial stewardship: coordinated efforts to reduce the unnecessary use of antibiotics in humans and animals with a special emphasis on last-resort antibiotics that are reserved for the treatment of highly resistant pathogens.
Antimicrobial susceptibility testing (AST): routine procedure in diagnostic laboratories to determine whether an identified pathogenic bacterium is resistant or sensitive toward an array of clinically relevant antibiotics.
Blood-cleansing: refers to the extracorporeal purification of blood in the context of BSI to selectively remove pathogenic microorganisms and toxic molecules secreted by such pathogens.
Bloodstream infection (BSI): infectious disease caused by viable pathogenic bacteria or fungi that replicate in the bloodstream of a patient. The infection often causes a strong inflammatory response that can result in septic shock.
Limit of detection (LOD): refers to the lowest concentration of bacteria or fungi that can be consistently detected by a diagnostic test with a 95% certainty.
Nanobodies: small antibody fragments corresponding to the variable domain of heavy-chain-only antibodies found in camelids. Owing to their small size of ~15 kDa, they are more robust than classical antibodies and can be inexpensively produced at large amounts.
Sensitivity: probability of a diagnostic test identifying true positives. Its value is calculated by dividing the number of true positives by the sum of all positive test results (including both true positives and false positives). A sensitivity of 100% means that all positive test results correspond to true positives and a sensitivity of 0% means that all positive test results correspond to false positives.
Specificity: probability of a diagnostic test identifying true negatives. Its value is calculated by dividing the number of true negatives by the sum of all negative test results (including both true negatives and false negatives). A specificity of 100% means that all negative test

Saccharomyces cerevisiae; various Gram-negative bacteria, such as *Escherichia coli*; numerous Gram-positive bacteria, including *Staphylococcus aureus*; fungi, as well as LPS, in standard *in vitro* assays [15–17] (Box 1).

results correspond to true negatives and a specificity of 0% means that all negative test results correspond to false negatives.

FcMBL-mediated capture was shown to be suited for **blood-cleansing**, thereby efficiently removing pathogens from the bloodstream [13,18]. Blood-cleansing was carried out by continuous blood circulation through an extracorporeal device reminiscent of the spleen, called an engineered biospleen, coated with FcMBL. In a BSI rat model, the device captured >90% of spiked *S. aureus* or *E. coli* from the blood within 1 h [13]. The blood-cleansing method was also used in combination with antibiotic therapy for better efficacy and simultaneous removal of released PAMPs [18] or endotoxins, which has been shown to improve survival rates in infected rats [13]. In addition, FcMBL was used to isolate *S. aureus* from clinical samples [15], and an FcMBL sandwich enzyme-linked lectin sorbent assay (ELLecSA) was shown to be useful for the detection of intact pathogens and PAMPs in whole blood from patients with sepsis [16,19].

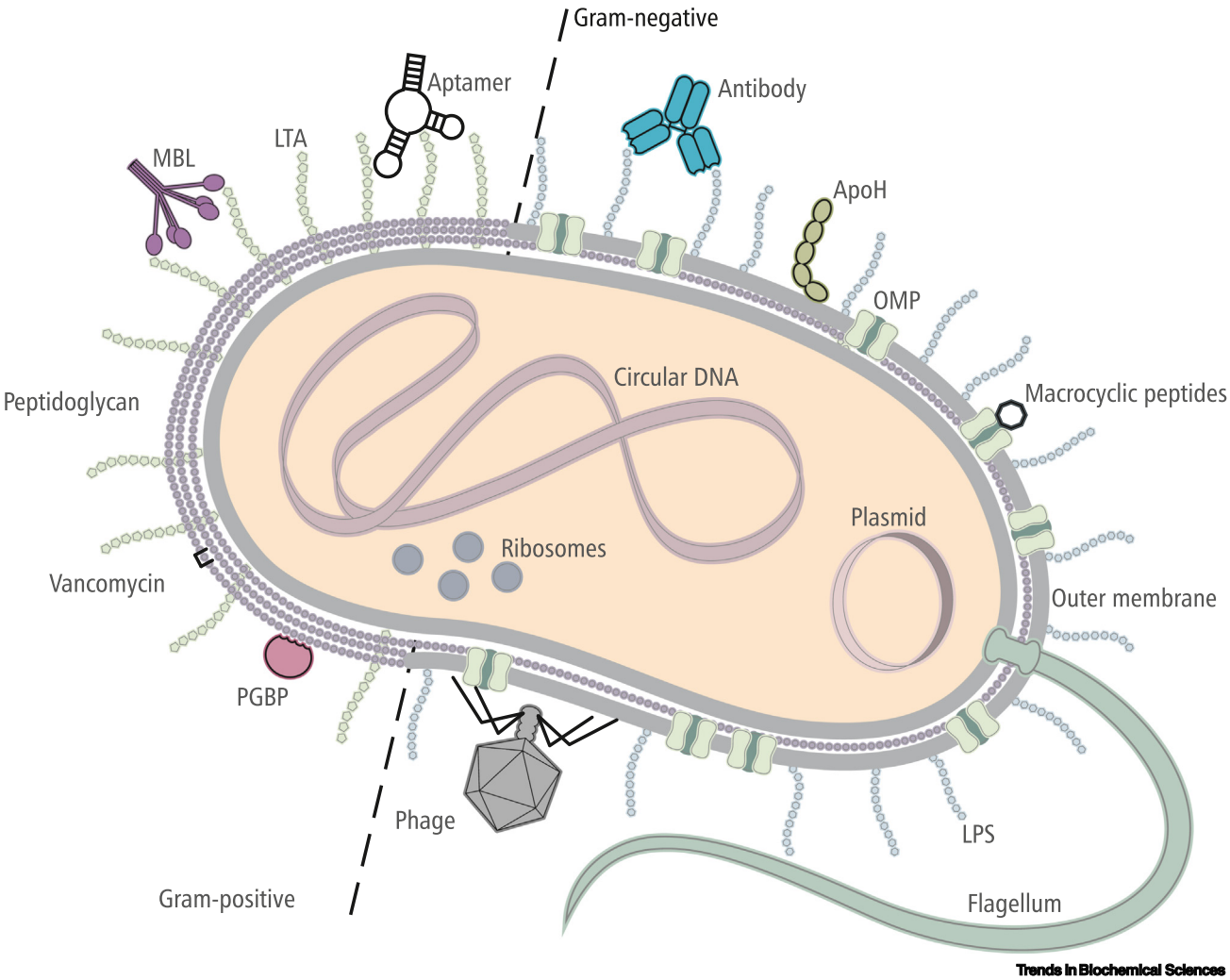

Figure 1. Overview of various biomolecules binding to the surface of Gram-positive (left) or Gram-negative (right) bacteria. Most affinity molecules bind to sugars, such as lipoteichoic acid (LTA), lipopolysaccharide (LPS), or peptidoglycan. Abbreviations: ApoH, apolipoprotein H; MBL, mannose-binding lectin; OMP, outer membrane protein; PGBP, peptidoglycan-binding protein.

The FcMBL ELLecSA assay showed high sensitivity (81%) and specificity (89%). Most recently, a recombinant human MBL coupled with magnetic beads was used to capture *Candida* spp. from blood, thereby enabling a substantial reduction of the turnaround time for *Candida* identification in clinical samples [17].

Apolipoprotein H

Apolipoprotein H (ApoH), also named beta 2-glycoprotein I (β2-GPI), is an acute-phase immune protein in plasma and strongly binds to negatively charged phospholipids, thereby acting as an LPS scavenger [20,21] (Figure 1). Activated ApoH binds a variety of sepsis-causing pathogens [22]. A system of ApoH-coated magnetic beads efficiently captured and concentrated bacteria from blood [21], allowing the detection of *E. coli* at 1 CFU/ml of blood using quantitative PCR. Live bacteria captured on beads may also be grown in culture, although ApoH has a narrower binding spectrum of pathogens compared with MBL [17].

Peptidoglycan-targeting biomolecules

Another capture technology exploits the strong affinity of peptidoglycan-binding protein (PGBP) for the peptidoglycan layer of Gram-positive bacteria (Figure 1). Engineered PGBP-modified magnetic nanobeads captured *S. aureus* and other Gram-positive bacteria in plasma in less than 1 h [23]. An additional promising candidate targeting peptidoglycan are lysozymes. These are small antibacterial enzymes produced by innate immune phagocytes that catalyze the breakdown of bacterial peptidoglycan, leading to the lysis of sensitive bacteria. A catalytically inactive lysozyme has been used to capture *S. aureus* and other bacteria from whole blood via a magnetic separation method [24].

Antibodies as highly specific biomolecules

Historically, polyclonal antibodies were generated against pathogenic bacteria by immunizing mice or rabbits with intact bacteria or bacterial extracts (Figure 2). These antibodies mostly targeted lipopolysaccharides (O-antigen) and flagellar proteins (H-antigen) and were used to characterize different serotypes of Gram-negative pathogens [25] (Figure 1). Carbohydrate

Box 1. Immunomagnetic separation as a method of choice to capture bacteria via biomolecules

Immunomagnetic separation (IMS) is the dominant method for the rapid enrichment of bacteria (Table I). Three main parameters have to be taken into consideration when establishing capture assays involving IMS: (i) bead size and bead chemistry; (ii) coupling of the biomolecule (direct chemical coupling versus biomolecular interaction); and (iii) orientation of the biomolecule (random versus uniform). Magnetic bead sizes used for bacterial capture range from 18 nm [88] to 2.8 μm [68], but, in most cases, they are in the range of 100–500 nm. Smaller beads decorate the captured pathogen, as is nicely visualized on scanning electron microscopy images [13,88]. In contrast to tiny beads, 2.8-μm magnetic beads have a similar size as the captured bacteria themselves, which might cause steric hindrance of binding. The main disadvantage of tiny beads is their comparatively small magnetic pull, although this can be overcome by adding larger uncoated superparamagnetic beads along with the smaller beads to facilitate efficient capture [13]. An advantage of smaller beads is that they are more compatible with microfluidics, whereas big beads might influence flow rates due to friction [13,89].

The two prevalent coupling methods are direct chemical coupling (covalent) or coating via affinity molecules (noncovalent). For example, highly stable aptamers were covalently and directionally coupled to amine-functionalized magnetic nanoparticles using a free carboxyl or amine group on those amines [57,58,88]. Similarly, vancomycin was covalently attached using its free carboxyl group to free amine groups of fluorescent polystyrene microbeads [90]. Proteinaceous biomolecules, such as antibodies, immune molecules, and phage proteins, were typically coated via affinity molecules (in most cases biotin/streptavidin) to ensure their biophysical integrity.

All biomolecules contain surfaces termed paratopes (including loops and/or binding cavities) that directly interact with the target molecule on the captured bacterium. Ideally, a high paratope density is reached after coupling, as this not only impacts the binding capacity of the beads, but may also result in favorable binding avidity effects. To assure that the paratopes are freely accessible, it is advisable to aim for a uniform orientation of capture biomolecules. If the biotin/streptavidin interaction is chosen for bead functionalization, the optimal method is to biotinylate the capture biomolecule with a single biotin at a site that is distant from the paratope [91]. In case of FcMBL, directionality was achieved by labeling an N-terminal and highly accessible lysine using amine-reactive biotin. The *Salmonella*-specific gp37-gp38 phage protein was biotinylated by introducing an Avi-tag for site-specific, enzymatic biotinylation [13,15,68]. Similarly, histidine-tagged peptidoglycan-binding protein was coupled to Ni-NTA biosensors and Ni-NTA magnetic beads of 900 nm to enable a uniform orientation [23].

Table I. Overview of capture biomolecules, target structures, and capture efficiencies

Biomolecule	Category	Capture method	Target	Target organism	Limit of detection	Efficiency	Capture duration	Refs
FcMBL	Immune protein	Magnetic beads (500 nm)	Sugar moiety (mannose, *N*-acetyl glucosamine)	Multiple (here *Staphylococcus aureus*)	$<10^4$ CFU/ml	85%	2 h	[15]
FcMBL	Immune protein	Superparamagnetic nanobeads (128 nm) combined with microfluidics	Sugar moiety (mannose, *N*-acetyl glucosamine)	Gram-negative and Gram-positive bacteria, fungi, endotoxins	Not determined (n.d.)	>90%	1.25 l/h for ~5 h	[13]
MBL	Immune protein	Nanoparticles (200 nm) in microfluidic channel with slanted ridge array	Sugar moiety (mannose, *N*-acetyl glucosamine)	Multiple (here *Escherichia coli*)	n.d.	>90%	0.6 ml/h	[89]
ApoH	Immune protein	Magnetic beads (200 nm)	LPS of many pathogens	Multiple (here *E.coli*, *Enterococcus gallinarum*, and *Candida tropicalis*)	1 CFU/ml (based on qPCR)	n.d.	30 min	[21]
Lysozyme	Enzyme	Magnetic beads (2.8 μm)	Peptidoglycan	Multiple Gram-positive bacteria (here *S. aureus*)	100 CFU/ml (based on qPCR)	94%	90 min	[24]
Peptidoglycan-binding protein	Immune protein	Magnetic nickel beads (1 μm)	Peptidoglycan	*S. aureus* and *Bacillus cereus*	~50 CFU/ml	80%	<1 h	[23]
α-PNAG antibody	Antibody (monoclonal)	Protein A-coupled Fe_3O_4 nanoparticles (300 nm)	Poly-*N*-acetyl glucosamine (PNAG)	Multiple (here *S. aureus*)	n.d.	>98%	5 min	[40]
α-MOMP sybodies	Nanobody (synthetic)	No capture tested but suited	Major OMP (MOMP) of *Legionella pneumophila*	*L. pneumophila* (reference strains for serogroups 1, 2, 6, and 12)	n.d.	n.d.	Unknown	[96]
α-*S. aureus* aptamer	Aptamer	Fe_3O_4-Ce6-Apt nanosystem; Apt-Fe3O4@mTiO2 magnetic particles	*S. aureus* cells, selected by Cell-SELEX	*S. aureus*	10–2000 CFU	≥80%	1.5 h	[52,58,88]
α-*E. coli* aptamer	Aptamer	Fe_3O_4-Ce6-Apt nanosystem; Apt-Fe3O4@mTiO2 magnetic particles	Mixed purified OMPs of *E. coli*	*E. coli*	10–2000 CFU	≥80%	1.5 h	[44,58,88]
α-*S. aureus* aptamer	Aptamer	Magnetic silica particles	*S. aureus* cells, selected by Cell-SELEX	*S. aureus*	~700 CFU	~80% (PBS) 60% in blood	Unknown	[48,57]
α-*Acinetobacter baumannii* aptamer	Aptamer	Magnetic particles	*A. baumannii* cells, selected by Cell-SELEX	*A. baumannii*	100 CFU/ml	n.d.	Unknown	[97,98]
cy(LLFFF)	Cyclic peptide	No capture tested but suited	MrkA	*Klebsiella pneumoniae*	n. d.	n. d.	Unknown	[85]
Vancomycin	Antimicrobial	Fluorescent microbeads (2 μm) on a microchip	Peptidoglycan precursors	Gram-positive bacteria	~10^5 CFU/ml	n.d.	2 h	[90]
Gp37-gp38 (long tail fiber)	Phage protein	Magnetic beads	OmpC and LPS	*Salmonella* spp.	10 CFU/ml	93±5%	45 min–2 h (ELLTA)	[68]

(continued on next page)

Table I. (continued)

Biomolecule	Category	Capture method	Target	Target organism	Limit of detection	Efficiency	Capture duration	Refs
Bacteriophage BCCP-T4	Entire phage	Streptavidin-coated magnetic beads	Unknown	*E. coli*	800 CFU/mL	70%	2 h	[76]
Gp048	Phage protein	Magnetic beads (2.8 μm)	Unknown	*Campylobacter jejuni*	100 CFU/ml	80%	3 h	[99]

antigens are located at the bacterial surface and, therefore, can be accessed easily by antibodies. However, carbohydrates are highly variable and, thus, it is challenging to find broadly specific antibodies. For *E. coli*, for instance, 181 O-antigens and 53 H-antigens have been described [26,27].

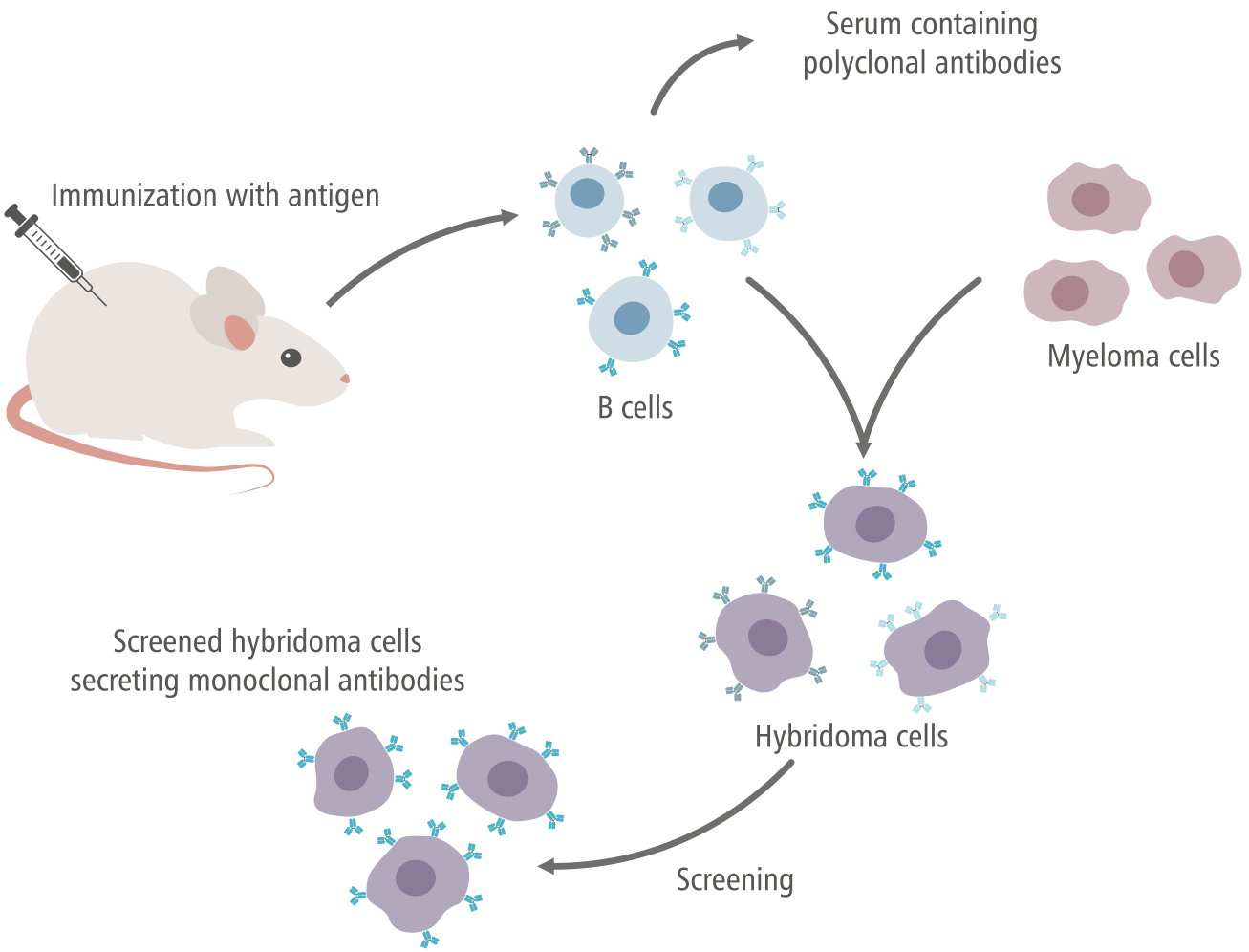

Figure 2. Antibody generation. The classical pipeline to generate antibodies begins with immunization of a mouse, followed by B cell isolation. Polyclonal antibodies are present in the serum of the immunized mouse. Hybridoma technology is used to generate monoclonal antibodies.

Diagnostic antibodies

Despite many pathogen-specific antibodies being available, they are not often used in routine diagnostics due to their limited species coverage (owing to binding to variable O-antigens/H-antigens), high LODs, and expensive production. Consequently, their use in the diagnostic laboratory has been restricted to difficult-to-grow intracellular bacteria, such as *Coxiella* spp., *Rickettsia* spp., or *Mycoplasma pneumoniae*, using immunofluorescence assays on fixed tissues or shell vial cultures [28,29]. While antibodies are rarely used in clinical microbiology, they are extensively used in the food safety industry to detect foodborne pathogens, such as *E. coli* O157:H7 [30,31].

Therapeutic monoclonal antibodies suitable for diagnostics

Therapeutic antibodies that bind to the bacterial surface can, in principle, be used for diagnostic purposes. Although the concept of therapeutic antibacterial antibodies was established more than 80 years ago after the historical success of serum therapy [32], modern usage of bacterial antibodies has been restricted to targeting bacterial toxins [33]. However, over the past 10 years, renewed interest in antibacterial monoclonal antibodies (mAbs; Figure 2) has emerged in response to AMR [34]. Consequently, an increasing number of clinical and preclinical programs with antibacterial mAbs have been initiated [25,35]. Among them is the human anti-*Pseudomonas aeruginosa* antibody directed against LPS serotype O11 [36] and a mAb directed against *Enterococcus faecalis* pili protein, the latter of which was successfully applied to indicate the site of enterococcal infection after radiolabeling and imaging [37]. Furthermore, poly-*N*-acetylglucosamine (PNAG) was identified as a highly conserved surface polysaccharide produced by bacteria, fungi, and protozoal parasites. A fully human mAb binding to PNAG showed opsonic and bactericidal activities against all PNAG-producing pathogens, including numerous Gram-positive and Gram-negative bacteria [38,39], and was shown to capture up to 98% of *S. aureus* within 5 min [40].

Aptamers are highly robust, nucleotide-based capture molecules

Aptamers are small single-stranded DNAs or RNAs of 25–100 nucleotides (5–40 kDa) that fold into a well-defined 3D structure (Figure 1). Compared with antibodies, the production of aptamers is inexpensive and has minor batch-to-batch variation. They are heat and pH stable and can return to their proper conformation after denaturation without loss of binding capacity. It is straightforward to chemically modify, label, and couple aptamers, making them highly suitable as carrier molecules for diagnostics. Libraries of distinct oligonucleotide sequences (10^{12}–10^{14} molecules) are used for *in vitro* selection in a process called systematic evolution of ligands by exponential enrichment (SELEX), which was invented in 1990 [41,42] (Figure 3). Traditional SELEX methods require a soluble and pure target, whereas, in newer developed SELEX methods, aptamers can bind their target in complex mixtures depending on the desired application [43,44]. Cell-SELEX selection is performed to select aptamers that specifically bind the target in the cellular context, including negative selections to enhance specificity for a bacterial species [45–48]. By contrast, Toggle-cell-SELEX is used to generate aptamers exhibiting broad crossreactivity to different bacterial species or genera by changing the target cell used in each reaction round [49,50] (Figure 3). As described later, aptamers have emerged as promising biomolecules, which permit magnetic enrichment at a low detection range without affecting bacterial viability. Therefore, they show the potential to be broadly used for rapid BSI diagnostics.

Aptamers targeting pathogenic bacteria

Numerous aptamer sequences recognizing an extensive array of BSI pathogens, including *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, and *P. aeruginosa*, have been published over the past 15 years. Whereas some aptamers are highly specific for a species (e.g., *E. coli* [51], *S. aureus*

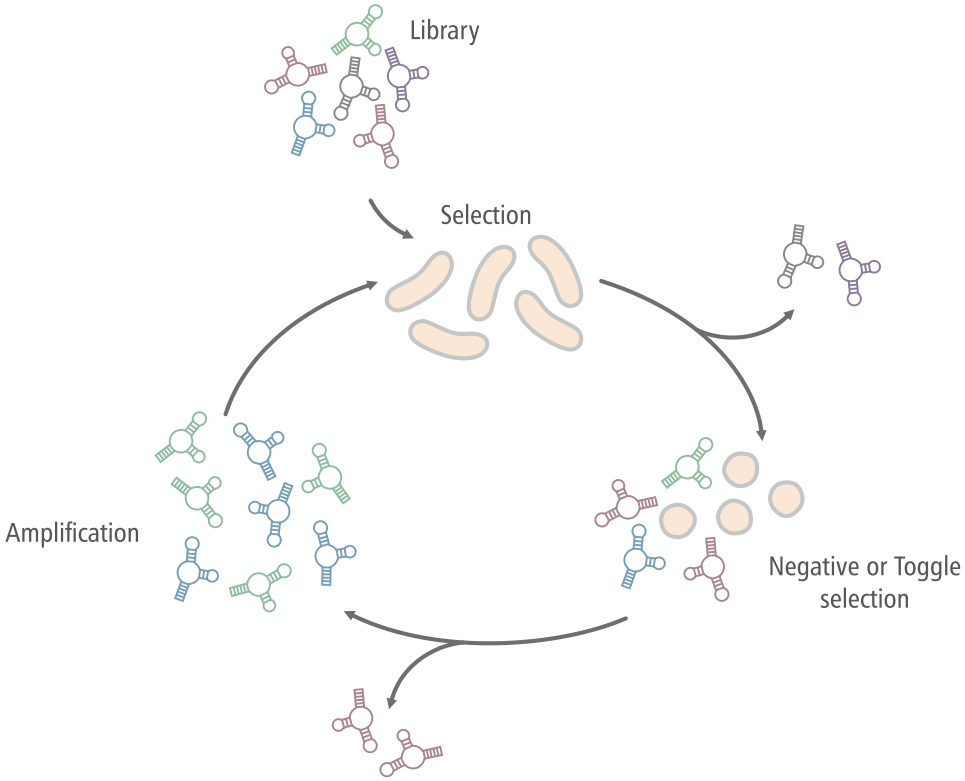

Figure 3. Aptamer generation. Libraries of distinct oligonucleotide sequences (10^{12}–10^{14} molecules) are panned against target cells using an *in vitro* selection method called systematic evolution of ligands by exponential enrichment (SELEX). Repeated selection rounds result in DNA molecules called aptamers that either bind specific pathogens (if CELL-SELEX is used) or a broad set of pathogens (if Toggle-cell-SELEX is used).

[52], or *P. aeruginosa* [53]) or a specific strain [e.g., *E. coli* (ETEC) K88 [54]], there are also aptamers with an expanded host range, recognizing up to six Gram-positive and Gram-negative species [50]. For bacterial detection, cellular localization, as well as affinity determination, aptamers are labeled with 5′-fluorescein. Some aptamers have also been radiolabeled with ^{99m}Tc to study scintigraphic images and identify *S. aureus* infectious foci in mice. This technique could be used to develop specific diagnostic radiopharmaceuticals for different infection types [55]. Typical affinities of aptamers are in the range of 10–100 nM. Except for the *E. coli* (ETEC) K88 aptamer [56], which had been selected against the purified K88 fimbriae protein, the target structures (proteins, lipids, or carbohydrates) of most antibacterial aptamers are unknown.

Coupling aptamers with nanoparticles

Aptamers have been coupled with a range of nanoparticles (NPs). For example, to capture bacteria from blood, silica NPs have been coated with aptamers against *S. aureus*. Capture efficiency in phosphate buffered saline (PBS) was 78%, and 61% in whole blood spiked with 10^2 CFU/ml [57]. Furthermore, the authors developed a fluorescent detection method called NanoKeeper, which can be activated by releasing micrococcal nuclease (MNase) from *S. aureus*. This sensitive assay provides a new and effective strategy to monitor *S. aureus* infections directly in blood samples [57]. Aptamers were also conjugated with mesoporous TiO_2-coated magnetic (M)NPs, which were used to capture *S. aureus* or *E. coli* spiked into blood with high enrichment efficiencies (80%) [58] (Box 1).

In another study, iron oxide MNPs were functionalized with chlorin e6 (Ce6) molecules and aptamers that recognized either *S. aureus* [52] or *E. coli* [44] (the so-called 'Fe_3O_4-Ce6-Apt nanosystem'). These magnetic beads enriched *S. aureus* from whole blood with high efficiency (>80%), whereas enrichment with the *E. coli*-specific aptamer system was <20%. Furthermore, Ce6 was used for extracorporeal blood disinfection by irradiating these molecules with infrared light, generating singlet oxygen molecules [59].

Some aptamers against *Salmonella* Enteritidis and *Salmonella* Typhimurium were shown to have antibacterial activity and, therefore, were not suited to capture live pathogens [46]. In most instances, however, aptamers do not have intrinsic bacteriostatic or bactericidal activities. Aptamers recognizing methicillin-resistant *S. aureus* (MRSA) coupled with gold nanorods and aptamers against *S. aureus* or *E. coli* immobilized on plasmonic gold film both induced cell death with targeted photothermal therapy (PTT) [60,61]. Conjugation of an aptamer to ampicillin inhibited biofilm formation and eradicated the already-formed biofilm of *Salmonella enterica* [62].

Exploiting the binding capacity of natural enemies of bacteria

Bacteriophages infect bacteria and archaea, replicate within their host cell, and destroy them by bursting and releasing newly formed bacteriophages for further infection. Identification and binding to their target bacteria are mediated through their receptor-binding protein (RBP) and have evolved to be highly specific. Given that bacteriophages have naturally evolved to kill bacteria, they often cannot be directly used to capture live bacteria. Instead, the isolated RBPs are used as engineered proteins for capture (see later).

Bacterial targets of phages

Depending on the bacteriophage family, RBPs are either located at their tail fibers, tail spikes, or central tail spikes for targeting their associated bacterial cell surface receptors [63] (Figure 4). These highly specialized bacteriophage proteins have evolved for binding receptors that are located on the cell surface of the host, such as LPS, glycosylated LTA, components of secretion machineries, flagella, pili, or outer membrane proteins (OMPs) [63,64] (Figure 1). The localization and density of these receptors have a pivotal role in the recognition process [65].

Compared with antibodies, bacteriophage host attachment has many advantages. Natural antibodies often bind to the most abundant surface molecules, which often cause the most significant immune response. However, bacteria can easily escape antibody detection by changing their surface-exposed epitopes. By contrast, surface epitopes recognized by bacteriophages are often more difficult to mutate. In addition, some bacteriophage RBPs bind with very high affinity to bacterial cells and are very stable proteins, whereas antibodies often exhibit moderate affinities and are less robust [66–68].

Most phages bind to LTA of Gram-positive or LPS of Gram-negative bacteria and, therefore, feature strain-specific infection patterns [69,70]. To overcome this natural limitation, synthetic phage libraries with mutated tail fibers can be used to screen for expanded host range and suppression of bacterial resistance to phage infection [69,70].

Use of phage proteins for bacterial capture

The use of entire phages as bacterial capture tools has two main disadvantages: (i) they have enzymatic activity and thereby basal lytic activity; and (ii) they have relatively large sizes compared with antibodies. Therefore, phage-encoded host interaction proteins, such as tail fibers, tail spikes, or central tail spikes, have been widely used as affinity molecules.

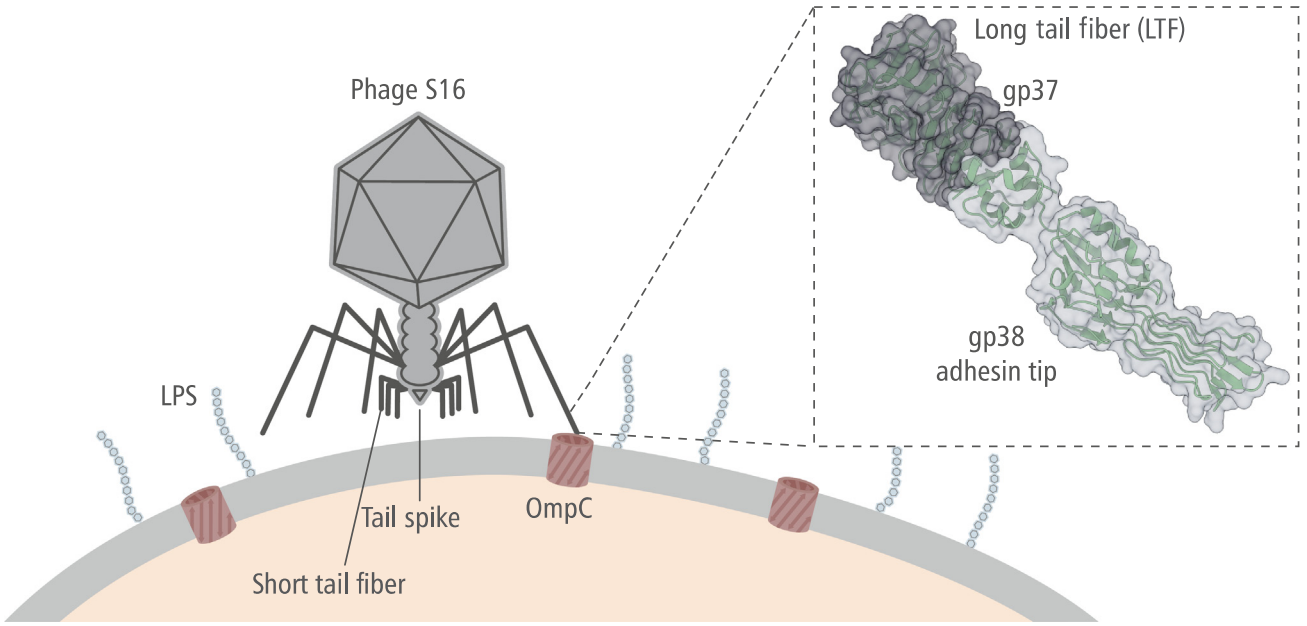

Figure 4. Molecular cloning of phage proteins. Bacteriophages bind bacterial surface molecules via long tail fibers (LTF), short tail fibers, or tail spikes. Cloning and recombinant production of these phage proteins afford pathogen-specific biomolecules, as shown by the example of the long tail fiber complex gp37-gp38 of the bacteriophage S16, which recognizes outer membrane protein C (OmpC) on the *Salmonella* cell surface.

For historical reasons, phages and phage proteins were mainly used to detect food- and water-borne pathogens. For example, the long tail fiber complex gp37-gp38 of the bacteriophage S16 recognizes OmpC on the *Salmonella* cell surface with nanomolar affinity (5.2 nM) [71,72] (Figure 4). Gp37-gp38 immobilized on magnetic beads identified all relevant *Salmonella* species and subspecies with high efficiencies (>97%) and, when coupled to horseradish peroxidase, this phage protein allowed for colorimetric identification of *S.* Typhimurium at concentrations down to 10^2 CFU/ml in only 2 h [68].

Reports of the use of phage proteins for clinical microbiology are still rare. In two studies, intact phages immobilized on surface plasmon resonance (SPR) chips were used for the detection of *S. aureus* (10^4 CFU/ml) or *E. coli* K12 (7×10^2 CFU/ml) [67,73]. Similarly, filamentous phages were immobilized on a gold surface of a magnetoelastic biosensor, and the response toward different *S.* Typhimurium ATCC1331 concentrations from either pure suspensions, water, or fat-free milk was measured with a detection limit of 5×10^3 CFU/ml [74,75]. T4 phages were also immobilized on magnetic beads and used to capture *E. coli* cells with efficiencies >70% using bacterial concentrations ranging from 10 to 10^5 CFU/ml [76] (Box 1).

Antimicrobial peptides as capture molecules

Antimicrobial peptides are produced by several microorganisms as complex natural defense molecules [77]. In addition, they are also artificially generated by means of chemical synthesis [78,79]. The main application of antimicrobial peptides is their use as anti-infective agents [80] as well as a treatment option to tackle drug-resistant pathogens [81,82]. Some antimicrobial peptides exhibit high affinities toward their bacterial target molecules and, therefore, have been used as capture molecules for diagnostic purposes.

Application of antimicrobial peptides for bacterial capture

Vancomycin is a broad-spectrum glycopeptide antimicrobial that recognizes the D-Ala-D-Ala moiety of the uncross-linked peptidoglycan precursor, thereby blocking the final steps in peptidoglycan synthesis (Figure 1). Vancomycin-modified NPs or magnetic beads have been observed to effectively and selectively capture Gram-positive bacteria and Gram-negative bacteria down to 10^5 CFU/ml [59,83]. Subsequent detection by surface-enhanced Raman scattering (SERS) allowed rapid culture- and label-free identification of bacteria in blood [59,83].

Oligoacyllysine (OAK) is a novel peptide-mimetic antimicrobial compound that has a high affinity for bacteria. It has been coupled with polystyrene beads and used to capture and concentrate bacteria [84], although it was designed for large volumes of environmental samples, such as water.

A macrocyclic peptide was generated in a recent targeted approach against the highly conserved MrkA protein of pathogenic *K. pneumoniae* [85] (Figure 1). The identified macrocyclic molecule cy (LLLFF) was observed to bind intact *K. pneumoniae* with high affinities of ~50 nM and, thus, shows potential for use as a capture molecule.

Concluding remarks

In light of the global AMR crisis and to adhere to antimicrobial stewardship programs, it is becoming increasingly important to treat bacterial infections with appropriate antibiotics. In the context of BSIs, rapid diagnostics is of particular importance, because it allows escalation or de-escalation of antibiotics treatment, depending on the outcome of the diagnostic test. The capture of viable pathogens from patient samples is a cornerstone to establish faster phenotypic diagnostic tests. Therefore, the past decade has witnessed increased publication activity in the realm of pathogen capture biomolecules.

As discussed herein, immune proteins, antibodies, aptamers, phage proteins, and antimicrobial peptides are the prevalent biomolecules developed for bacterial capture (see Outstanding questions). Interestingly, these biomolecules originate from diverse scientific fields, likely explaining their current use for specific applications. For example, the immune molecule FcMBL is mainly propagated as a central part of an engineered biospleen used for extracorporeal blood-cleansing of patients critically ill with sepsis [13]. Antibodies were mainly generated as future therapeutic products to treat chronic infections of, for example, *P. aeruginosa* [36]. Aptamers are popular in nanotechnology because they can be treated like chemicals due to their stability [58]. Last but not least, phage proteins have been mainly developed in food microbiology and safety [64]. For these reasons, the winner of the race to be the predominant capture biomolecule in the context of clinical microbiology is not yet decided.

For future applications in BSI diagnostics, the broad-range detection ability of immune proteins, such as FcMBL and ApoH, appears to be a significant advantage over other molecules, which target only a single species or (in the case of antibodies) only a few strains of a species. However, more thorough biochemical and biophysical studies are needed to compare these 'super-bullet' immune molecules side-by-side with more specific but much narrower antibodies, aptamers, and phage proteins. In addition, it will be interesting to see whether the broad-range interactions of immune molecules come at the price of unspecific interactions, by which contaminant and undesired cell types are dragged along during the capture reaction.

Another critical question is how the capture biomolecules of the future will be generated. Whereas immune proteins have been described as part of the innate immune system and simply need to

Outstanding questions

Bacterial capture molecules greatly differ in their specificity. Very narrow specificities (recognition of only few strains of a bacterial species) are impractical, while broadly specific biomolecules, such as immune proteins, appear more promising. Does low specificity come at the price of the unspecific binding of undesired cell types and molecules?

There are different experimental strategies for how bacterial capture molecules are identified, generated, and engineered. Which binder identification strategies will finally dominate the field?

Bacterial capture biomolecules are highly diverse in terms of size, stability, affinity, and the way they are produced. Which biophysical and biochemical features of capture biomolecules will be most relevant for their implementation as capture tools in diagnostics?

be cloned and produced, the generation of antibodies and aptamers, as well as the discovery of phage proteins, is a major technical challenge. For aptamers, there is a trend toward untargeted selections against intact cells. This strategy appeared to be successful in expanding the host range of aptamers but comes at the price of the target structure being unknown. In terms of phage proteins, their significant advantage is that bacteriophages and their targeted bacteria coevolved over very long periods and, therefore, are often highly robust and potent biomolecules. However, it resembles a fishing expedition to find phage proteins that strongly bind to bacterial pathogens because it is impossible to predict the target structure on the bacterial surface. Antimicrobial peptides offer a straight-forward functionalization, in particular when they are chemically synthetized. By contrast, many of them are likely too toxic and, therefore, will not allow live capture of pathogens, while others do not exhibit the required affinity to allow for efficient pathogen enrichment.

For targeted approaches, antibodies and antibody fragments offer highly advanced technologies. However, in classical antibody generation with intact cells or cell debris, the major epitopes are highly variable carbohydrates. This makes full coverage of even a single species extremely challenging. Therefore, an attractive targeted approach is to raise antibodies (or antibody fragments) against conserved, purified OMPs and, in a second step, identify binders that recognize the target in the context of the living cell. In a proof-of-concept study to generate **nanobodies** against the major OMP of *Legionella pneumophila*, this strategy has recently been demonstrated to be feasible (Box 2).

Box 2. An innovative pipeline to generate nanobodies against conserved outer membrane proteins

Targeting conserved OMPs of Gram-negative pathogens with biomolecules is a promising strategy to capture live bacteria. A prominent example is the long tail fiber complex gp37-gp38 of the bacteriophage S16, which recognizes OmpC on the *Salmonella* cell surface and achieves high genus coverage [71,72]. However, phage proteins recognizing OMPs are rare, and antibodies usually target surface-accessible molecules, such as O-antigens, because OMPs are deeply buried in the protective LPS layer and, thus, are not readily accessible by antibodies due to the large size of the latter. By contrast, nanobodies are considerably smaller than antibodies and have a demonstrated track record of being able to bind to buried epitopes of membrane proteins [92].

Nanobodies can be selected against purified OMPs from immune or synthetic libraries using ribosome and phage display [93–95] (Figure I). This allows the generation of binders against highly conserved OMPs to achieve high species coverage. Thus, the advantages of high species coverage (as is the case for some phage proteins targeting OMPs) and the rational choice of target structures and target cells (as is the case for antibody generation) are combined. However, only a small subset of epitopes accessible during selection against detergent-purified OMPs can also be reached in the cellular context.

To overcome this screening bottleneck, a technology was established called NestLink, which enables the deep-mining of pre-enriched nanobody pools for rare binder candidates that bind to the targeted OMP in the cellular context [96] (Figure I). At the core of NestLink are DNA-encoded peptide barcodes designed to be optimally detected by mass spectrometry, which are genetically fused to a pre-enriched nanobody pool in a process called library nesting. Through the concerted action of next-generation sequencing and mass spectrometric analysis of flycodes, several-thousand binder candidates can be efficiently screened for desired biophysical properties, such as OMP binding in the context of a Gram-negative pathogen.

NestLink was validated in the context of targeting the major OMP (MOMP) of *Legionella pneumophila*. In a first step, synthetic nanobodies (sybodies) [93] were generated against the detergent-purified MOMP of *L. pneumophila*. ELISA revealed several binders that recognized detergent-purified MOMP but failed to bind in the cellular context [96]. In a second step, a total of 1444 unique sybodies were fused to around 24 000 flycodes, and the entire pool was deep-mined via pull-downs using intact *L. pneumophila* cells for binders that recognized MOMP in the cellular context. The subsequent mass spectrometric analysis revealed five sybodies (out of the 1444 unique ones screened), which specifically bound MOMP in the context of *L. pneumophila*.

In summary, the combination of nanobody and sybody selection with the deep-mining capacity of NestLink constitutes a robust technology platform to generate highly specific and affine nanobodies that bind any targeted OMP in the context of Gram-negative cells.

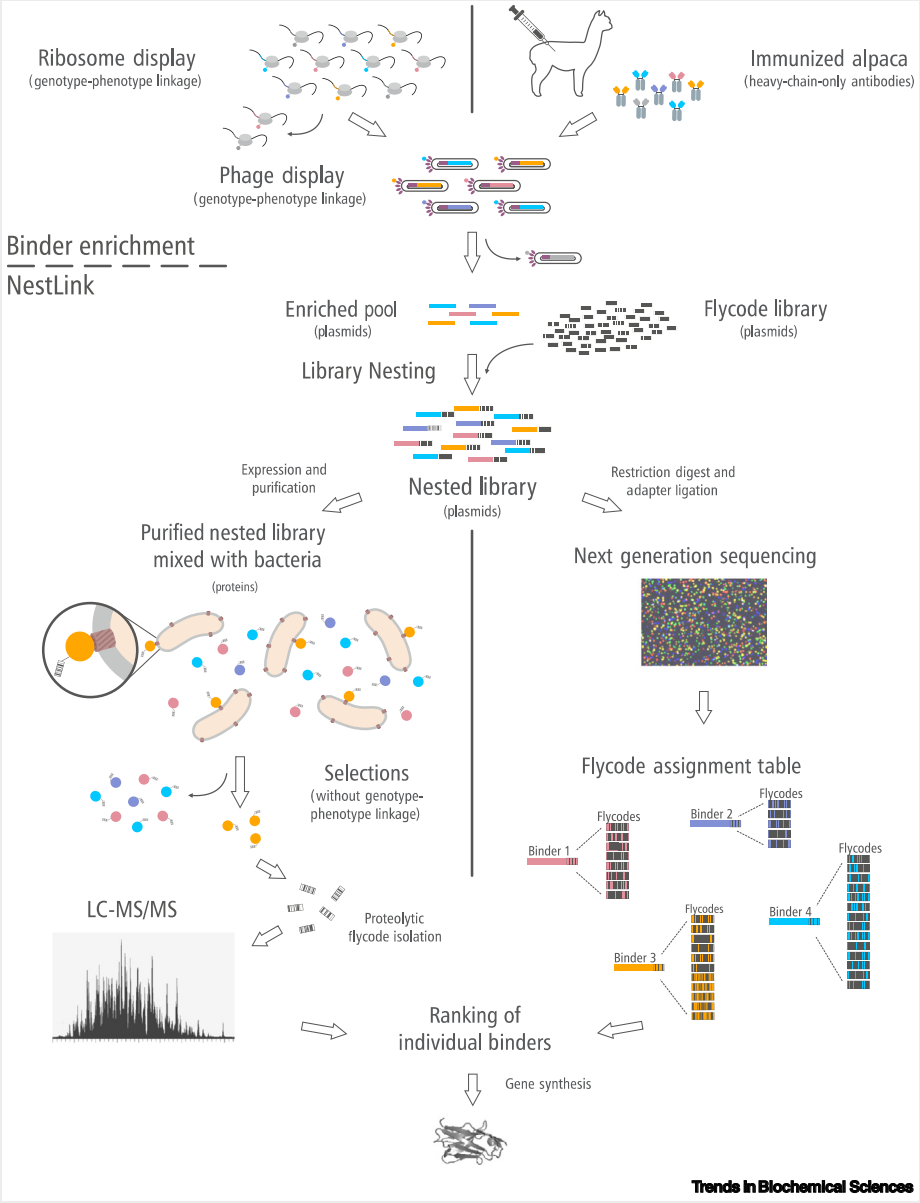

Figure I. Technology platform to generate nanobodies against conserved outer membrane proteins (OMPs) of Gram-negative bacteria. In a first step, nanobodies are enriched against a purified target OMP. To this end, a camelid is immunized with the OMP. Alternatively, one round of ribosome display is performed with a synthetic nanobody (sybody) library. Next, the nanobodies of the immunized camelid or the ribosome display output are cloned into a phage library, which is used to further enrich nanobodies against the OMP. In a second step, NestLink is performed. NestLink begins with library nesting, in which the enriched nanobody pool (typically 2500 binder candidates) is cloned in-frame with the flycode library, a genetically encoded peptide barcode library designed to be optimally detected by mass spectrometry. The nesting reaction is performed such that each binder candidate is tagged with, on average, 20–30 different flycodes. Next-generation sequencing (NGS) is performed to assign flycode sequences to the sequences of the unique binder candidates. The nested nanobody library is expressed and purified as a pool, followed by a pull-down with intact bacteria containing the target OMP at the cellular surface. Subsequently, the flycodes attached to the target-binding nanobodies (orange circle) are isolated by proteolytic cleavage and analyzed by liquid chromatography coupled with mass spectrometry (LC-MS/MS). Using the information obtained from NGS, nanobodies that recognize the target OMP in the cellular context are ranked and, finally, their clonal sequence is obtained by gene synthesis.

Finally, the implementation of future rapid diagnostic methods in the daily routine is a huge challenge. First, the new methods need to offer a clear medical benefit. Hence, it does not only matter whether the new diagnostic method is simply faster, but it is also equally vital that it yields critical information (e.g., resistance profiles of relevant pathogens) that can inform a decision made by the physician (e.g., escalation or de-escalation of antibiotic therapy). In particular, the accuracy of the diagnostic finding in terms of sensitivity and specificity are crucial in this context. Second, diagnostics is a cost-sensitive market, which affects future products in two ways: (i) the consumables for a kit and the associated machines should not be too expensive; and (ii) in the diagnostic laboratory, the technical procedure should be simple, with short hands-on time and high throughput. In light of these requirements, highly stable biomolecules that are cheap to produce and have a long shelf life (e.g., aptamers or nanobodies) appear ideal.

In light of the pressing AMR crisis, there is an urgent need for rapid diagnostic tests. However, growth-dependent enrichment of pathogens such as by blood culturing, for example, is inherently slow and, therefore, impedes the development of faster diagnostics. The efficient capture of pathogens via biomolecules described in this review offers an attractive route to overcome growth-dependent bottlenecks in diagnostic pipelines. Importantly, capture via such biomolecules does not kill the enriched pathogens, which facilitates both genetic and phenotypic analyses of the isolated pathogens. Thus, capture approaches become attractive in particular in the context of miniaturized AST, involving, for example, microfluidics [86] or nanomechanical sensors [87].

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Declaration of interests

M.A.S. is co-founder and shareholder of Linkster Therapeutics AG. L.M.H. is employee of Linkster Therapeutics AG.

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