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REVIEW

Phagemid Vectors for Phage Display: Properties, Characteristics and Construction

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Phagemids are filamentous-phage-derived vectors containing the replication origin of a plasmid. Phagemids usually encode no or only one kind of coat proteins. Other structural and functional proteins necessary to accomplish the life cycle of phagemid are provided by the helper phage. In addition, other elements such as molecular tags and selective markers are introduced into the phagemids to facilitate the subsequent operations, such as gene manipulation and protein purification. This review summarizes the elements of the phagemids and their corresponding functions. Finally, the possible trends and future direction to improve the characteristics of the phagemids are highlighted.

Introduction

Phage display, invented by Professor Smith in 1985,[1] has attracted much attention because of its power and simplicity. As the main tools used in phage display, filamentous phage f1, fd and M13 (Ff phages) are very stable under a variety of harsh conditions used for selection of phage binders including extreme pH, high temperature,[2] presence of DNase, proteolytic enzymes[3] or nonaqueous solution.[4] Unique and other structural and genetic characteristics of the filamentous phage make phage display an extremely powerful tool for bioengineering, for example, screening ligands, developing new drugs, designing vaccines, evolving molecules, diagnosing diseases, drawing the genetic maps, delivering targeted drugs or biosensing.[5–7] Numerous proteins or peptides with high specificity and affinity have been isolated from phage display libraries using affinity selection (biopanning) and widely used in different fields.[8–11] To date, many books[12–17] and comprehensive reviews[18–20] aimed at illustrating the principles of phage display, procedures of constructing libraries, biopanning strategies and applications of the technology are available. However, there are no reviews that would systematically introduce and analyze the characteristics and functions of phagemids in details. This review focuses on some aspects of phagemids, including

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Abbreviations used: DARPin, designed ankyrin-repeat protein; IG region, intergenic region; phOx, 2-phenyl-5-oxazolone; ssDNA, single-stranded DNA; RF, replicative form; scFv, single-chain variable fragment; MCS, multiple cloning site; mAb, monoclonal antibody.

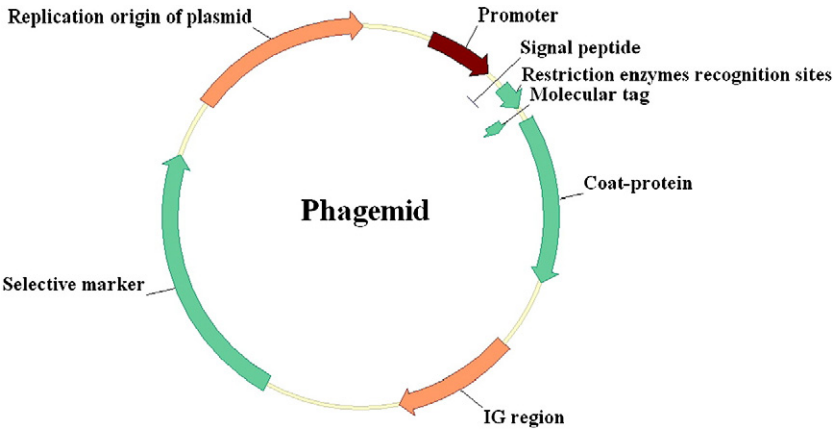

Fig. 1. The scheme of phagemid vector.

structure, morphology, advantage, life cycle in phage display, the elements and corresponding functions, which would give some guidelines for choosing phagemid vectors and constructing new ones. The possible trends and future perspectives of phagemid vectors are also discussed in this review.

Biology of Phagemids

Structure and morphology of phagemids

Phagemids are Ff-phage-derived vectors, containing the replication origin of a plasmid. The basic components of a phagemid mainly include the replication origin of a plasmid, the selective marker, the intergenic region (IG region, usually contains the packing sequence and replication origin of minus and plus strands[21]), a gene of a phage coat protein, restriction enzyme recognition sites, a promoter and a DNA segment encoding a signal peptide (Fig. 1). Additionally, a molecular tag can be included to facilitate screening of phagemid-based library. The functions of these elements will be discussed in Construction Strategies and Characteristics of Phagemids in detail.

Phagemids can be converted to filamentous phage particles with the same morphology as Ff phage[22] by co-infection with the helper phages,[23] such as R408,[24] M13KO7[25] and VCSM13 (Stratagene). As with other filamentous phages, the length of progeny phage particles would be varied along with the length of phagemid DNA.[26]

Life cycle in phage display

The phagemid itself is not able to finish the assembly of progeny phage particles independently, and the life cycle in phage display is summarized in Fig. 2. First, the phagemid particles containing the DNA fragment encoding a foreign protein infect the F′ strain of *Escherichia coli*. Upon entering the host cell, the single-stranded DNA (ssDNA) of phagemid particles can be converted into replicative form (RF) of the phagemids by the host RNA and DNA polymerases and topoisomerase, which act on the replication origin of minus strand located in the IG region of the phagemids. Due to the origin of a plasmid, the RF of phagemids could replicate normally in the host cell like a plasmid used in the recombinant DNA research. Usually, these origins used in the phagemids are ColE1[27] or p15A, which allows to produce many RF of phagemids in the host cell and enough templates for the production of the fusion proteins. After transcription and translation, the fusion proteins are delivered to the inner membrane of host cells followed by removal of a leader signal peptide by signal peptidase and then folded. A Ff phage replicative origin of plus strand, present in the IG region of phagemid, is not activated until the host cells are superinfected by helper phage,[23] which provides genes of phage proteins missing in a phagemid. Since the helper has its own origins of minus or plus strands, the replication proteins act not only on the phagemid but also on the helper phage itself. However, due to the defect in origins of the helper phage, phage ssDNA is produced in much smaller amount than phagemid ssDNA. Finally, the packing sequence, also located in the IG region of phagemids, interacts with the assembly complex formed by pI, pIV, pXI and host cell thioredoxin. After elongation and termination, the progeny phages of phagemids are mature, and the life cycles of phagemids are accomplished. As for the function and structure of IG region in phagemids, it is similar with that in filamentous phages. For more details, please consult the references elsewhere.[21,25,28–31]

Advantage of phagemids

Phages and phagemids are the most common vectors used in phage display, while phagemids are used more widely than phages due to the reasons

summarized below.[32,33] First, genomes of phagemids are smaller and can accommodate a larger foreign DNA fragment. Second, the phagemids are more efficient in transformation that allows obtaining a phage display library with high diversity. Third, a variety of restriction enzyme recognition sites are available in the genome of phagemids convenient for DNA recombination and gene manipulation. Fourth, the expression level of fusion proteins can be controlled and modulated easily. Finally, phagemids usually are genetically more stable than recombinant phages under multiple propagations.

Construction Strategies and Characteristics of Phagemids

To date, many strategies and genetic elements have been adopted for different purposes in developing phagemid vectors. In the first application of phagemid vectors for phage display, Bass *et al*. constructed phagemid phGH-M13gIII by using helper phage M13KO7 and plasmid pBO473.[32] In phGH-M13gIII, human growth hormone was fused to the carboxy-terminal domain of truncated pIII.[32] Later, many types of phagemid vectors, including type III and type VIII phagemids, harboring *gpIII* or *gpVIII*, have been constructed, which are presented in Tables 1 and 2. The basic elements of phagemid and their functions in phage display are discussed below.

Coat proteins

Regarding the type of coat proteins used to display foreign proteins, phagemids can be divided into type III, type VI, type VII, type VIII or type IX, which contain the coat protein pIII (or its truncated form), pVI, pVII, pVIII or pIX, respectively. Among these types, type III and type VIII phagemids are the most commonly used vectors in phage display. Phagemids of type III and type VIII that are usually used for phage display are listed in Tables 1 and 2. As for other types of phagemids, there have been only a few publications reported so far.[85,86]

The main differences between type III and type VIII phagemids are the length of foreign peptides displayed, the density or the copy number of the displayed foreign proteins in the progeny phages and the influence of infection of progeny phages.[32,34] Compared with type VIII phagemids, the type III phagemids can accommodate larger foreign proteins. As indicated in Fig. 3, for larger proteins, such as the anti-2-phenyl-5-oxazolone (phOx) single-chain variable fragment (scFv), the type III phagemid vectors were preferred compared with that of the type VIII.

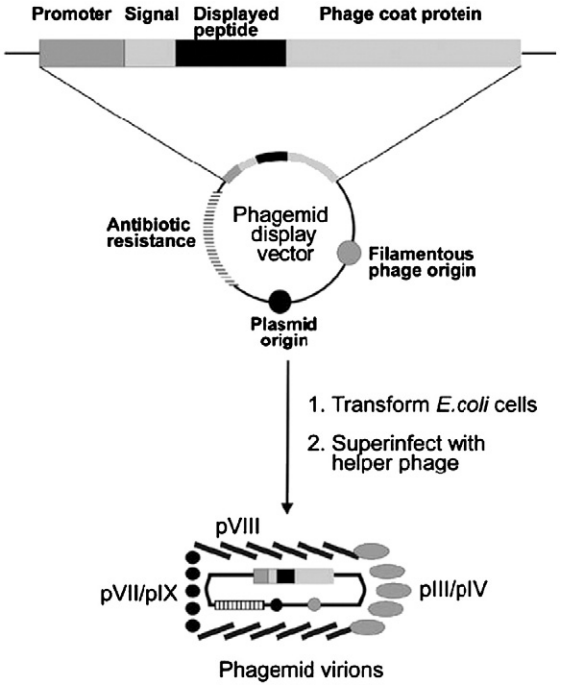

Fig. 2. Phagemid display vector. A "typical" phagemid display vector contains origins of replication for double-stranded DNA and ssDNA synthesis (plasmid and filamentous phage origins), an antibiotic resistance gene providing selection of transformed bacteria and a fusion gene under the control of a regulated promoter. If the fusion gene derives from phage gene VIII or III, a signal sequence fused to the coat protein directs secretion of the coat protein and is subsequently cleaved by signal peptidase, leaving the coat protein spanning the inner membrane. Phagemid vector is converted into infective phage by superinfection of phagemid-bearing cells with helper phage, as described in the text. Reprinted with permission from Ref. 21.

In a phage displaying system using type III phagemids harboring the phage gene *III*, there are less than five copies of fusion proteins in the progeny phages due to structural properties of the Ff phages. Only when there is no gene *III* in the genome of helper phage would five copies be present in the progeny phages.[89] However, the progenies of type VIII phagemid can append much more than five copies of foreign proteins,[90] even hundreds or thousands of copies, which are determined by the genome size of phagemids when the gene *VIII* of helper phage is missing. With the increase in the copies of displayed foreign proteins in this displaying system, the length of displayed peptides would decrease drastically to a few or a dozen amino acids.[91] Because of multiple copies of displayed proteins, it is probable that the avidity effect, but not the affinity, is dominant, which makes it difficult to distinguish the proteins with different affinities.

Table 1. Type III phagemids and their properties

Phagemid	Promoter	Signal peptide	Useful restriction sites	Molecular tags	Codon TAG	Reference
phGH-M13gIII	*P*lacZ	stII	ApaI, XbaI	N	N	32
pSEX	P_{T7}	pelB	PstI, BamHI	Trypsin cleavage site	N	33
pComb3	*P*lacZ	pelB	XhoI, SpeI	N	N	34
pHEN1	*P*lacZ	pelB	PstI, SalI, XhoI, NotI	c-*myc*	Y	35
phGHam-g3	*P*ap	stII	NR	N	Y	36
pFAB-4	*P*lacZ	pelB	SfiI, NotI	Trypsin cleavage site	N	37
pFAB-5c	*P*lacZ	pelB	SfiI, NotI	Trypsin cleavage site	N	37
pMKFabGene3	*P*lacZ	pelB	NotI, XbaI	HA-tag	N	38
pCANTAB3His_6	*P*lacZ	gIII	ApaL1, PstI, XhoI, NotI	myc, His_6	Y	39
pCANTAB5His_6	*P*lacZ	CAT	ApaL1, PstI, XhoI, NotI	myc, His_6	Y	39
pCANTAB6	*P*lacZ	CAT	ApaL1, PstI, XhoI, NotI	myc, His_6	Y	39
pCANTAN5E	*P*lacZ	gIII	SfiI, NotI	E-tag	Y	Pharmacia
pComb3H	*P*lacZ	Omp A	SacI, XbaI, NcoI, XhoI, SpeI, SfiI	N	N	40
pB3	*P*lac	gIII	NotI, NcoI	myc	Y	41
pKN1	*P*lacZ	Omp A	MluI, NheI, XhoI	Albumin-binding protein	Y	42
pComb3B	*P*lacZ	pelB	XhoI, BstXI	N	N	43
pHEN1-hFo	*P*lacZ	pelB	PstI, SalI, XhoI	myc	Y	44
pHEN1-hJu	*P*lacZ	pelB	PstI, SalI, XhoI	myc	Y	44
pLPscH	*P*lacZ	Lipoprotein	NotI	myc, His_6	Y	45
pFab-5c.His	*P*lacZ	pelB	SfiI, NotI, XhoI, Sal I, NcoI, AscI	Trypsin cleavage site, His_6	N	46
pto2H10a3s	*P*lac	Omp A	NR	FLAG, myc	Y	47
pLUCK	*P*lacZ	pelB	NotI, NcoI,	myc	N	48
pCW99	NR	NR	lox P	myc	NR	49
pHEN4	*P*lacZ	pelB	SfiI, NotI	HA-tag	Y	50
pK100	*P*lac	pelB	SfiI	myc, FLAG	Y	51
pSEX81	*P*lac	pelB	PvuI, NotI	Trypsin cleavage site	N	52
pV1	*P*lacZ	pelB	PstI, SalI, XhoI, NotI	FLAG	Y	53
pAS38	NR	Omp T	NR	NR	NR	54
$pC3^-$	*P*lacZ	pelB	XhoI, SpeI	N	N	55
pTP127	*P*lacPO	pelB	BglII, XbaI	N	N	56
pDN322	*P*lacZ	pelB	PstI, SalI, XhoI, NotI	D_3SD_2-FLAG-His_6	Y	57
pRPLS/Fab1	*P*tac	pelB	NheI, AscI, SfiI, NotI	NR	NR	58
pRlac3	*P*lac	gIII	MCS	NR	NR	59
pRpsp2	*P*psp	pelB	MCS	NR	NR	59
pCES1	*P*lac	gIII, pelB	ApaL1, PstI, XhoI, NotI, Asc I, SfiI, BstEI	myc, His_6	Y	60
pCANTAB5X	*P*lacZ	gIII	SfiI, NotI, XbaI	E-tag	Y	61
pFUW80	*P*lac	pelB	XhoI, SpeI, XbaI, SalI, EcoRI	His_5	Y	62
pCANSS	*P*lac	NR	SfiI	T_7-tag, E-tag	NR	63
pDAN5	*P*lacZ	Bacterial leader sequence	BssHII, BspEI, SalI, Xho I, KpnI, NheI	SV5-tag, His_6	Y	64
pS1602	*P*tac	maltose binding protein	NsiI	NR	N	65
pMAB29	*P*lac	pelB	SfiI, XbaI, MunI, NotI	myc	N	66
pMAB77	*P*lac	pelB	SfiI, XbaI, MunI, NotI, NheI	His_6	N	66
pMAB87	*P*lac	pelB	SfiI, XbaI, MunI, AscI, SalI	His_6	Y	66
pMAB66	*P*lac	pelB	SfiI, XbaI, MunI, NotI, NheI	His_6	N	66
pCANTAB5L	*P*lacZ	gIII	XbaI, StuI, SalI, KpnI	E-tag	Y	67
pHG-1m	*P*lac	NR	ApaLI, SfiI, SalI, NotI	myc, His_6	Y	68
pHEN6	*P*lacZ	pelB	SfiI	HA-tag, His_6	Y	69
pMID21	*P*lac	NR	ApaLI, AscI, SfiI, XbaI, BstEII, NotI	myc, His_6	NR	70
pHAL1	*P*lacZ	pelB	NheI, MluI, NcoI, NotI, HindIII, PstI	strep-tag	Y	71
pHAL2	*P*lacZ	pelB	NheI, MluI, NcoI, HindIII, PstI	strep-tag	Y	71
pHAL5	*P*lacZ	pelB	NheI, MluI, NcoI, NotI, HindIII	strep-tag	Y	71
pHAL6	*P*lacZ	pelB	NheI, MluI, NcoI, HindIII	strep-tag	Y	71
pY03-FLAG	*P*lac	NR	NcoI, MroI	E-tag, FLAG	Y	72
pChick3	*P*lac	pelB	NcoI, SalI	myc, His_6	Y	73
JSC	*P*lac	pelB	MCS	E-tag	NR	74
pMod1	*P*lac	gIII	MCS	myc, His_6	Y	75

N, not containing codon TAG; Y, containing codon TAG; NR, not reported.

Table 2. Type VIII phagemids and their properties

Phagemid	Promoter	Signal peptide	Useful restriction sites	Molecular tags	Codon TAG	Reference
PC89	*P*lac	gVIII	EcoRI, BamHI	N	N	27
pKfdH	*P*tac	gVIII	HpaI	NR	NR	76
pIF4	*P*lac	pelB	HindIII	NR	NR	77
pCBAK8	*P*lacZ	RBS-pelB	XhoI, XbaI	NR	NR	78
p8V2	P_{BAD}	gVIII	MCS	NR	N	79
pComb8	*P*lac	pelB	XhoI, SpeI	NR	NR	Barbas, C. F.
pS1607	*P*tac	Maltose binding protein	NsiI	NR	N	65
pS657a	*P*tac	Maltose binding protein	NsiI, XbaI	NR	Y	65
pEGFP-lacp8	*P*lac	gVIII	EcoO109I	NR	N	80
pM1165a	*P*tac	Maltose binding protein	NsiI	NR	N	81
pTat8-CD147	*P*lac	Tor A	XhoI, SpeI	NR	NR	82
Tag-pGP8	*P*tac	Pho A	XhoI, KpnI	FLAG	NR	83
pA2	P_{BAD}	NR	EcoRI, BglI	NR	NR	84

N, not containing codon TAG; Y, containing codon TAG; NR, not reported.

Therefore, the type VIII phagemids are used in phage display in order to expand the scope of the candidate ligands, and the type III phagemids are used to reduce or eliminate the avidity effect for selection of high-affinity proteins.

Besides that, the N-terminal domain of coat protein III is involved in infecting host cells. To infect *E. coli*, filamentous phages use a filamentous bacterial appendage known as F pili, which bring phage pIII tip to the TolA protein on the bacterial surface. If the type III phagemids are used, the fusion proteins containing the whole protein III synthesized in the host cell can affect the regeneration of host cells' F pili and prevent infectivity of helper phages. As a result, the progenies of phagemid can be eventually affected. Therefore, in type III phagemids, such as pK100, pMAB66, amino acids 250~406 in the C-terminus of wild-type protein III are usually used as anchoring proteins.[51,66] On the contrary, there are no similar problems in type VIII phagemids.

Multiple cloning sites

For fusion of foreign proteins with coat proteins, the nucleic acid fragments coding for corresponding foreign proteins need to be integrated into genes of coat protein by means of DNA recombination to allow their transcription and translation. There are two strategies to achieve this aim. In the first strategy, the foreign gene and the coat protein gene are linked together and then integrated into the genome of a miniphage that only contains the replication origins of the plasmid and phage IG regions. In another one, the foreign genes are directly cloned into the phagemid vectors. No matter which method is used, the gene manipulation needs to be carried out on both the vectors and foreign proteins genes. Therefore, the presence of endonuclease recognition sites facilitates the insertion and integration of foreign protein genes.[92]

Wild-type phages Ff are not commonly used as vectors as they usually do not have appropriate restriction enzyme recognition sites in the vicinity of the coat protein genes. Many vectors have already been developed using site-directed mutagenesis to introduce single restriction enzyme recognition sites or eliminate redundant ones to facilitate insertion of foreign coding fragments and other genetic manipulations. In addition, restriction endonuclease recognition sites can also be introduced into the upstream and downstream of coat protein coding fragments by the PCR method. Thus, when the coat protein coding fragments are integrated into the miniphages, the restriction endonuclease recognition sites can be simultaneously inserted into the genome of the miniphages.[32] In most cases, a variety of restriction sites were put together to provide multiple cloning site (MCS) for convenient manipulations. For example, a 51-base-pair stuffer containing MCS from plasmid pBluescript was integrated into the genome of phagemid pComb3′ to obtain phagemid pComb3. As a result of these manipulations, the additional useful restriction sites in phagemid pComb3 allow for convenient genetic restructuring operations.[34] There are many other phagemid vectors, whose characteristics can be improved by eliminating, adding or transferring the MCS from other plasmids, such as pCBAK8,[78] pHEN1,[35] pCANTAB6,[39] pCANTAB5X[61] and pCANTAB5L.[67]

Some elements for preparation of soluble foreign proteins

Although genetically engineered phages can be used themselves as epitope mimics, substitute antibodies or drug carriers,[17] the ultimate goal of most researchers using phage display is to prepare peptides and proteins suitable for studying their behavior in relation to other molecules. In these cases, soluble foreign proteins with good

characteristics are often preferred. The common elements integrated in phagemid vectors for preparing soluble foreign proteins are discussed below.

Amber codon TAG

The amber codon TAG can be inserted between foreign genes and coat protein genes to enable the foreign proteins to be expressed as soluble form by changing the host strains. For example, in *E. coli sup*E strains such as XL-1 Blue MRF′, TG1, DH5aF′, ER2738, ER2537 and 16C9, TAG codon is recognized as glutamic acid codon instead of stop codon. As a result, the foreign protein is expressed as a fusion protein presented on the surface of progeny phages. In the non-suppressor strains such as TOP10F', JS5 and HB2151, TAG is recognized as stop codon that terminates translation at C-terminus of foreign proteins and allows expression of the foreign proteins in a soluble form. The *sup*E strains are usually used for presenting the foreign proteins or peptides. Individual phage displaying fusion proteins can be used to infect the non-suppressor strains for preparing the soluble proteins. For instance, Hoogenboom *et al*. constructed phagemid pHEN1 in which amber codon TAG was inserted between foreign protein genes and protein III gene. In this way, they achieve the goal of obtaining foreign proteins as soluble fragments by changing the host from suppressor to the non-suppressor strain HB2151.[35] In practice, site-directed mutagenesis is one of the most commonly used methods to introduce an amber codon TAG between the foreign protein genes and coat protein genes in constructing new phagemid vectors.

Restriction endonuclease recognition sites

Very often, the target foreign proteins are required to be expressed as soluble proteins without changing vectors and host strains after they are obtained in fusion form by biopanning. In this case, the genome of individual phage can be digested by the corresponding restriction enzymes to remove coat protein genes and convert the phage display vectors into expression vectors for synthesis of soluble foreign proteins in the periplasm or cytoplasm of host cells. The coat protein genes can be removed by digestion with two different restriction enzymes. For instance, *gpIII* could be excised from pComb3 by digestion with restriction enzymes SpeI and NheI.[34] The *gpIII* in phagemids pFAB4[37] and pFAB5c[37] can be also removed by two different restriction enzymes. Sometimes, only one kind of restriction enzyme is employed. For example, Ames *et al.* added a NheI recognition site to each end of *gpIII* in phagemid vector, which improved and simplified removing of the *gpIII*.[38] Using a similar strategy, Hoet *et al*. constructed phagemid vector pMID21, in which the restriction enzyme MluI recognition sites were located in the upstream and downstream of *gpIII*.[70] Then digested by MluI and linked by T4 DNA ligase, the connection of sticky ends will induce a deletion of *gpIII* in the phagemid vector pMID21.[70]

Terminator

Terminator is another element for preparing soluble proteins. After the last round of biopanning, the terminator can be inserted between foreign protein genes and coat protein genes to prevent coat protein genes from being transcripted and translated. Takekoshi *et al*. inserted the universal translation terminator sequence into the genome of phagemid to prevent foreign protein Fab from

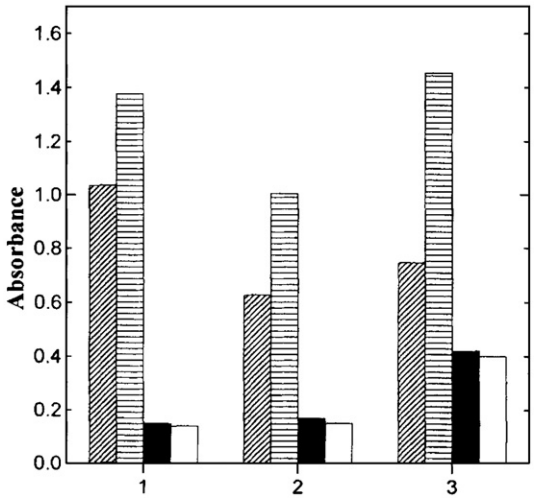

Fig. 3. ELISA of phage displaying the anti-phOx scFv attached to different viral coat proteins. Phage (6.5×10^9 transducing units) rescued from cells harboring pTK3552/A (wide-type pIII, diagonals), pTK3552/B (truncated pIII, horizontals), pTK3552/C (wide-type pVIII, filled bars) or pTK3552/D (IL-113∷wide-type pVIII, no fill) were added to 96-well MaxiSorp immunoplates (Nunc) coated at pH 8.1 with saturating amounts (10 μg) of phOx covalently coupled to bovine serum albumin (BSA), as described earlier by Nakela *et al*.[87] The anti-M13 ELISA was substantially performed according to the manufacturer's instructions: (1) Pharmacia-ELISA, (2) Stratagene anti-M13-serum, (3) 5′→3′-ELISA. With the Stratagene rabbit anti-M13 serum color, development was performed with a goat anti-rabbit Ab conjugated to horseradish peroxidase (Bio-Rad). When applying 3.3×10^{10} transducing units of the pVIII constructs, we measured absorbance of 0.20 (pTK3552/C) or 0.10 (pTK3552/D) units above the background values. The nonspecific background signals as determined with BSA were 0.14 absorption units for the Pharmacia and Stratagene systems and 0.41 units with the 5′~3′ kit. A SD of 10% ($n=3$) was generally obtained. Reprinted with permission from Ref. 88.

Table 3. Molecular tags and properties

Molecular tags	Sequence	Corresponding reagents	Phagemids
c-*myc*	EQKLISEEDLN	mAb 9E10	pHEN1[35]
Albumin-binding protein	46aa	BSA	pKN1[42]
T_7	MASMTGGQQM	Anti-T_7 mAb	pCANSS[63]
SV_5	GKPIPNPLLGLDST	SV5-P-K	pDAN5[64]
HA	YPYDVPDYGS	Anti-HA mAb	pMKFabGene3,[38] pHEN4,[50] pHEN6[69]
FLAG	DYKDDDDK[a]	Anti-Etag mAb	pto2H10a3s,[47] pV1,[53] pY03-FLAG[72]
Strep-tag II	SAWRHPQFGG	Streptacin	pHAL1,[71] pHAL2,[71] pHAL5,[71] pHAL6[71]
His_6	HHHHHH	Ni^{2+}	pCANTAB3*his*$_6$,[39] pCANTAB5*his*$_6$,[39] pFUW80,[62] pHEN6[69]
E-tag	GAPVPYPDPLEPR	Anti-Etag mAb	pCANTAB 5E, pCANSS,[63] JSC[74]

[a] The amino acid sequence could be different in phagemid vectors, such as sequences DYKD in pto2H10a3s[47] and DYKDDDDK in pV1.[53]

fusing with coat protein and to obtain soluble Fab using phagemid vector pRPLS/Fab1.[58]

Protease cleavage site

In general, soluble proteins can be obtained from fusion phage simply by digestion with protease. Compared with methods described in Amber codon TAG, Restriction endonuclease recognition sites and Terminator, protease cleavage is a simpler way for preparation of soluble proteins that does not need extra complicated procedures, such as changing a host for phagemids containing amber codon TAG or extraction of DNA, digestion with restriction enzyme, linkage by T_4 DNA ligase and transformation, which are required for phagemids with restriction enzyme sites and terminator sequence.

The general procedure in utilization of protease cleavage site for obtaining soluble proteins is summarized below. The DNA sequence encoding a protease cleavage site is cloned into the MCS when a phagemid vector is constructed. After biopanning, the soluble proteins could be prepared by treatment of the target phage particles with a desired protease such as trypsin. It should be mentioned here that the inhibitors of protease are often added to the solution of libraries and washing buffer to prevent undesired protease digestion.

Phagemid vectors such as pSEX,[33] pFAB4,[37] pFAB5c[37] and pFAB-5c.His[46] are constructed with the protease cleavage site, which can simplify the steps of preparing soluble proteins. Besides that, the binding phages could be removed effectively and moderately from the solid phase by incubation with trypsin in biopanning procedures.

Molecular tags

A molecular tag peptide that is recognized by common analytical methods could be fused to the C-terminus or N-terminus of a protein by recombinant DNA technology without affecting the function of the corresponding protein. In many fields, fused tag peptides have been use to detect, separate and purify corresponding proteins. Molecular tags that are common in phagemid vectors are listed in Table 3.

Usually, the DNA fragment encoding a molecular tag is inserted between foreign gene and coat gene in a phagemid vector. Except for functions mentioned above, it could also be used to evaluate the copy number of the displayed foreign proteins on the surface of individual phage or determine their affinity and specificity for targets, for example, by using ELISA and corresponding antibodies against the molecular tags. At the same time, they allow checking of whether a frame shift occurred during the integration process.

Promoters

The strength of the promoters required for gene transcription affects the expression level of fusion genes. The promoters commonly used in phagemid vectors include lactose promoter,[32] arabinose promoter,[84] alkaline phosphatase promoter,[36] phage shock promoter[59] and T7 promoter.[33] The expression of foreign proteins under the control of lactose promoter and arabinose promoter can be inducible by IPTG or lactose and arabinose, respectively. As the result of the induction, the level of foreign proteins appended to the surface of progenies can be modulated with different concentrations of inducers. Besides that, some promoters have additive functions. For example, Lowman *et al*. introduced alkaline phosphatase promoter in phagemid vector phGHam-g3.[36] With this vector, the fusion protein could be maintained at low level in *sup*E strains with medium containing high concentration of phosphate and for producing monovalent hGH-phage particles and screening for the high-affinity mutant; on the other side, the soluble hormone could be maintained at high level in non-suppressor strains by its growing in medium containing low concentration of phosphate without additional inducers.[36] Beekwilder *et al*. constructed phagemid vector pRpsp2 by using phage shock promoter, whose notable feature was that it could be activated by Ff phage protein IV.[59] Thus, under normal growth conditions without phage

protein IV, the promoter was suppressed[59] and was not induced until the helper phages that could provide protein IV superinfected the host cells. Besides the satisfactory control of the fusion protein synthesis, the use of the phage shock promoter does not require removal of glucose from the medium before superinfection.[59]

Signal peptides

Proteins would not fulfill their roles and functions until they are delivered or translocated to the correct position in host cells. Obviously, the fused foreign proteins also abide by these rules. Assembly of Ff phages occurs in the host cell membrane. Therefore, regardless of using pIII or pVIII display system, the fusion proteins need to be transferred to the host membrane to complete the assembly of progeny phages. Host cells have many different ways and secretion systems to sort and secrete proteins, six secretion systems being discovered by now. The use of signal peptide is undoubtedly one of the simplest ways to satisfy requirements of phage protein translocation through the bacterial membrane and their assembly in phage particles.[93]

Quite a few signal peptides have been used in phagemid vectors. In addition to pIII signal peptide and pVIII signal peptide, the majority of the commonly used signal peptides are ones from outer membrane proteins, such as pelB leader,[34,35,37] stII signal sequence,[36] CAT leader,[39] Omp A signal peptide,[42] lipoprotein signal sequence,[45,47] signal peptide of Omp T,[54] bacterial leader sequence,[64] signal peptide of maltose binding protein,[65,81] leader sequence of TorA[82] and signal peptide of PhoA.[83]

Since different secretion systems in host cells can direct processing of proteins or peptides, the signal peptides are very crucial elements for enhancing the level of display. As illustrated in Fig. 4, the display levels of designed ankyrin-repeat proteins (DARPins) were improved by changing the signal peptides from Sec to signal recognition particle dependent. Therefore, for some proteins or peptides that display poorly or at low level on the surface of phage, changing the signal peptides to use other translocation pathway was a good choice for promoting the display level.

Selective markers

Phagemids can endow the infected host cells with some traceable properties if they contain some selective markers. The first selective marker that was introduced into a phage-based vector is the α-fragment of β-galactosidase, whose encoding DNA sequence was cloned into the IG region of M13 phage

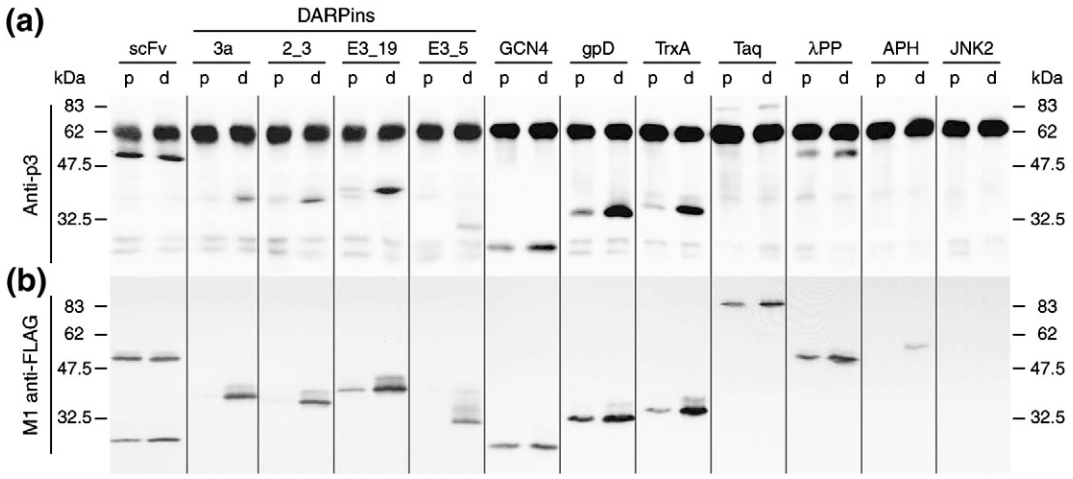

Fig. 4. Display yields of the various peptides or proteins of interest on phage particles. (a and b) CsCl-purified phage particles produced by the use of the respective phagemid indicated and normalized by UV absorbance to the same number of phage particles were separated by SDS-PAGE, blotted onto polyvinylidene fluoride membranes and detected with antibodies specific for the C-terminal domain of protein III (anti-pIII) (a) or the FLAG-tag (M1 anti-FLAG) located at the N-terminus of the peptide or protein of interest (b). The abbreviated names of the polypeptides are indicated on top of the lanes. The display yields are compared for each polypeptide using either the PhoA signal sequences (lanes labeled "p") or the DsbA signal sequence (lanes labeled "d") translocating the corresponding fusion protein by the Sec pathway or the signal recognition particle pathway, respectively. The molecular masses of marker proteins are indicated in kilodaltons at both sides of the blot. The band at 62 kDa in the anti-pIII blot corresponds to wild-type pIII and provides an additional normalization of the amount of phage particles loaded. Wild-type pIII is well known[94] to run at a seemingly higher molecular mass. Note that DARPin E3_5-pIII fusion runs at an apparently lower molecular mass than other DARPin fusions. This is also observed for this very stable protein in nonfused form, for which mass spectrometry indicated the expected mass, and may indicate incomplete denaturation on the SDS gel, compared to the less stable E3_19, which runs at the expected molecular mass. scFv is the abbreviation of scFv gp_D. Reprinted with permission from Ref. 95.

genome.[26] β-Galactosidase is a hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides. This enzyme can be split in two fragments, LacZα and LacZΩ, none of which is active by itself but both spontaneously reassemble into a functional enzyme. When a specific host cell is infected by the phage M13mp1, the small LacZα peptide is encoded by the phage M13mp1, while the large LacZΩ is encoded by the bacterial chromosome. As a result of this reaction called α-complementation, the infected host strain is able to use lactose as the only carbon source, indicating that α-complementation occurs.[26] Since then, many selective markers such as ampicillin resistance,[96] histidine operon *hisOGD*,[97] tetracycline resistance[98] and kanamycin resistance[25] have been adopted as the selective markers in developing phagemid vectors. Most of these selective markers are integrated into the IG region of the phagemid genome.

Besides the elements mentioned above, other elements and factors have also been cloned and considered to be adopted in phagemid vectors to improve their properties. For instance, the adoption of a strong transcription terminator can reduce the toxicity of foreign proteins to the host cells due to their leakage expression[47] and keep the phagemid stable.[56]

Future Perspectives

Based on different applications and requirements, many phagemid vectors have been constructed not only for screening foreign proteins but also for detecting, purifying and preparing proteins. With the development of the phage display technology, specialized phagemid vectors have been constructed to meet additional requirements. The copies of foreign proteins appended to the progeny phages, the length or molecular weight of the foreign peptides to be displayed and the distribution of foreign proteins appended on the surface of progeny phages are limited by the structure of Ff phage and the characteristics of foreign proteins. Up to now, only a few foreign proteins could be displayed on phages, which is far from meeting the needs of using phage display technology in the fields of proteomics research, bio-pharmacology, chemical evolution and biomaterials. Therefore, many improvements in phagemid vectors are needed to be done to solve the problems mentioned above. In view of the flaws and the structure of phage, phagemid vectors can be further improved in the following areas.

Improvement of the quality of phagemid-based phage display libraries

Some important characteristics, such as the presence of restriction enzyme recognition sites, nature of signal peptide, mode of gene expression and ability to track foreign proteins, should be considered when a phagemid vector is constructed. In particular, when it is adapted for the phage display libraries construction, it is their diversity that mostly determines the quality of a library.

The size and diversity of the libraries can be increased using recombination of different DNA fragments. Sblattero and Bradbury constructed phagemid vector pDAN5, which contains the lox sequence. As indicated in Fig. 5, with the help of Cre recombinase, the V_H and V_L genes from different phagemids were exchanged. Therefore, the diversity of the library was greatly improved.[64]

Since phagemid vectors are not infectious and cannot enter the host cell, they must be transformed into competent cells to produce phage particles.

Cen *et al*. cloned λ phage packaging signal sequence *cos* into the phagemid vector to construct phagemid pCZ4, able to be packaged *in vitro*.[99] This strategy increased the transformation efficiency and reduces the influences on diversity of library, which was caused by low conversion efficiency.[99]

Development of dual-display systems or vehicles

The dual-special reagents, which have specificity and affinity toward two different targets, are needed in many fields, such as molecular labeling and molecular imaging. The structure and characteristics of Ff phage allow their use as a basis for developing dual-special or multi-special reagents. Theoretically, pIII, pVI, pVII, pVIII and pIX are all able to anchor foreign proteins to achieve the goal of dual- or multi-display. pIII and pVIII are taken as examples. In a phage displaying system using type VIII phagemids harboring gene *VIII*, the pIII of helper phages and pVIII of phagemids are modified to achieve dual-display. As a result, the recombinant pIII of the progeny phages is provided by helper phage. Some of the pVIII is provided by phagemids in recombinant form, while others are provided by helper phage in wild-type form. As mentioned above, this system will affect the infectivity of progeny phage. Similar to the above system, the pVIII of helper phages and pIII of phagemids are modified in a similar phage displaying system but used type III phagemids harboring gene *III*. As a result, there are both wild-type pIII and recombinant pIII in progeny phages, which can reduce the impact of foreign proteins on infectivity of progeny phages.

In a phage displaying system using type III phagemids harboring gene *III*, Guo *et al*. modified the helper phage M13KO7 to integrate amino acid sequence VSGSSPDS (which could bind gold nanoparticles) into the N-terminus of protein VIII. The phagemid containing the gene of monoclonal antibody (mAb) 8G3 could recognize the spores of

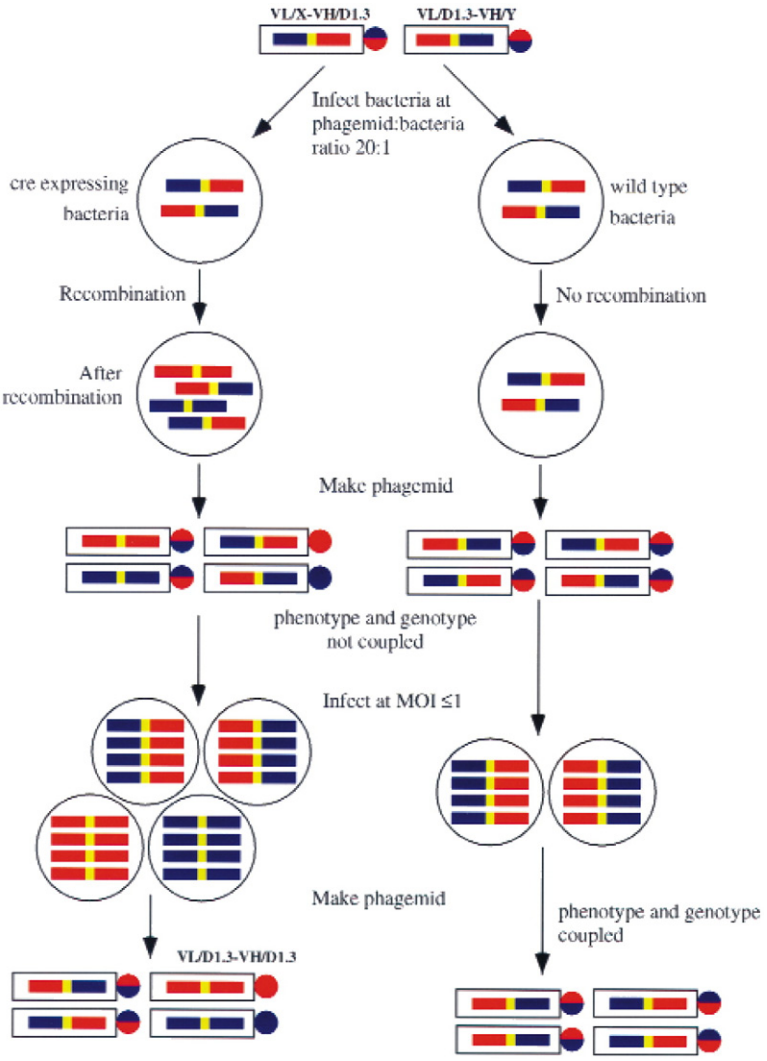

Fig. 5. The scheme of D1.3 recombination experiment. Two phagemids containing V_L/X-V_H/D1.3 and V_L/D1.3-V_H/Y (where X and Y represent irrelevant V genes) are added to either Cre-expressing bacteria or wild-type DH5aF at a multiplicity of infection of 20:1. After overnight growth, phagemid is made, reinfected into DH5aF′ at a phagemid:bacteria ratio of <1 (to couple genotype and phenotype) and tested by PCR and ELISA for the presence of functional V_L/D1.3-V_H/D1.3. Reprinted with permission from Ref. 64.

Bacillus anthracis. After being rescued by the modified helper phage, the bi-functional progenies could be produced (as indicated in Fig. 6a). As a result, the progeny phages that carried two different foreign proteins could recognize two corresponding targets, gold nanoparticles (as indicated in Fig. 6b) and spores of *B. anthracis* (as indicated in Fig. 6c and d) at the same time.[100]

Sunbul *et al*. used the similar strategy to achieve dual display in progeny phages, but by modification of protein pIII of the helper phage.[101] Gao *et al*. constructed vector pCGMT-1b in which pIX and pVII were used as anchor proteins for dual display. This strategy does not affect the infectivity of progeny phages.[86,102]

Development of new molecular tags

At present, most of molecular tags used in phagemid vectors are peptides. The detection of the fusion proteins labeled with these tags must rely on the tag-specific antibodies and respective substrates. New molecular tags that do not need extra reagents to detect will broaden the application fields of phagemid vectors.

The green fluorescent protein, which becomes fluorescent at a certain wavelength, can be used as molecular tags in real-time, rapid detection systems. Recently, Velappan *et al*. successfully appended the enhanced green fluorescent protein to the phage surface.[103] This approach may promote the discovery and development of new molecular tags.

Exploration components of other secretion systems

Given the life cycle of filamentous phage, the displayed proteins must be transferred to the inner member of host cell. Currently, signal peptides are used in phagemid vectors to transport the fusion

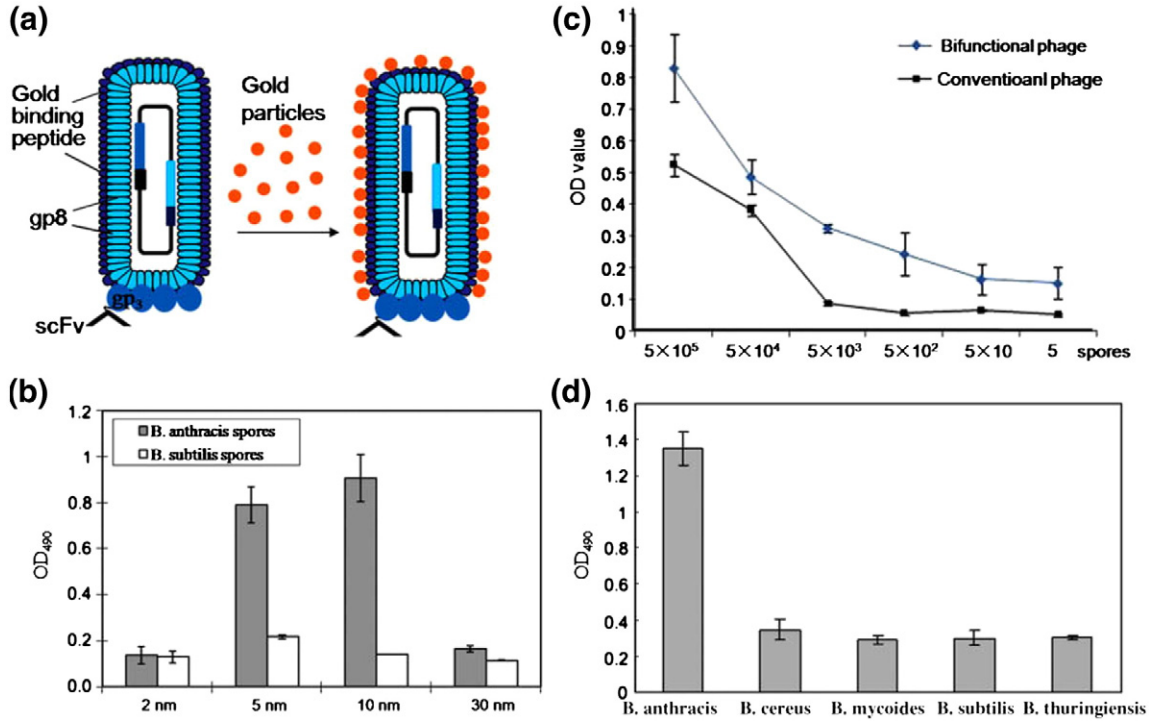

Fig. 6. Bifunctional phage-based immunoassay. (a) Scheme of bifunctional phage. (b) Analysis of the gold nanoparticle size for the optimization of the binding capacity to bifunctional phage. (c) Detection sensitivity of bifunctional phage immunoassay with silver enhancement and phage ELISA for *B. anthracis* spores. The polystyrene microtiter plate was coated with 100 μL of 10-fold serial dilutions of *B. anthracis* spores. For the bifunctional phage immunoassay with silver enhancement, the development of silver was monitored spectrophotometrically at 490 nm. For the conventional phage ELISA, 3,3′,5,5′-tetramethylbenzidine was used for color development. The reaction was stopped with 2 M H_2SO_4, and absorbance was determined at 450 nm. (d) Specificity of bifunctional phage-based immunoassay. *B. anthracis* and *Bacillus* control strain spores *Bacillus cereus*, *Bacillus mycoides*, *Bacillus subtilis* and *Bacillus thuringiensis* were examined by using the same method. Reprinted with permission from Ref. 100.

proteins to cell membrane. However, there are many other secretion systems in the host cells that are independent from the signal peptides, such as the type I[104] or type III[105] secretion system. Exploring these secretion systems or components involved for developing new phagemid vectors can bring new opportunities in phage display and present cytoplasmic proteins or the low display proteins.

Summary

Phagemid vectors are indispensable tools in phage display technology. Many phagemid vectors have been constructed according to the different requirements and objectives of experiments, which have greatly promoted the applications of phage display. However, these phagemid vectors can only be used to display a small portion of the proteins and cannot meet the requirements of some special purposes, such as real-time monitoring of displayed multiple-type foreign proteins. In short, improvements are still needed in developing phagemid vectors. It can be envisioned that phagemid vectors will play more important roles in the fields of biosensor, molecular recognition, protein interactions, drug development, molecular imaging and molecular evolution.

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