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(54) **Title**: MODIFIED BACTERIOPHAGE, BIOSENSOR CONTAINING SAME, AND METHOD OF USE

(57) **Abstract**: The invention relates to bacteriophages comprising a modified coat protein capable of binding to a metal oxide surface. The invention also relates to methods for detecting and/or quantifying targets in a sample using same.

Modified bacteriophage, biosensor containing same, and method of use

[FIELD OF THE INVENTION]

This invention is directed to the field of biosensors and more particularly to bacteriophage having a modified coat protein.

[BACKGROUND OF THE INVENTION]

Surface design for biologically oriented micro- and nano-electronic structures is currently shifting from chemically modified synthetic surfaces towards interfacial layers of genetically engineered biomaterials, like polypeptides, nucleic acids or other macromolecules [1]. This new approach is motivated by the fact that traditional molecular linkers, like thiol- or silane-based self-assembled monolayers, are relatively non-specific in their reactivity with the inorganic surface, and generally do not convey specific biorecognition properties to the synthetic micro- and nanoscale devices [2]. On the contrary, genetically engineered polypeptides have recently been shown to exhibit specific affinity to several Si-based inorganic surfaces [3,4], on top of their specific molecular recognition and genetic activity [5].

An essential aspect of biosensor development is the search for specific molecular linkers (mediators) for the attachment and/or recognition of the targeted biological species in solution. While thiol- and silane-based self-assembled monolayers are generally being used as mediators, the use of bacteria and phages has recently been reported as well, since they can be genetically engineered to exhibit specific affinity with several metallic or Si-based inorganic surfaces.

For integrated biosensing devices, it was recently shown that metallic oxides may represent significant advantages over metallic or silicon oxides thin films, since it provides for a more reliable passivating encapsulation of the device in some of the corroding biological media encountered for diagnostic applications [6].

[SUMMARY OF THE INVENTION]

The inventors have now found that specific foreign peptide sequences may modify a filamentous phage, when inserted in a coat protein of said phage, in order to convey to the latter a specific affinity towards metallic oxide surfaces.

The invention thus relates to bacteriophages comprising a coat protein modified with at least one amino acid sequence, wherein said amino acid sequence comprises 4 to 17 amino acids, and wherein the modified coat protein is capable of binding to a metal oxide surface.

In one embodiment, said amino acid sequence comprises 5 to 12 amino acids, more preferably 8 amino acids.

Typically, said amino acid sequence is inserted in place of native amino acids of a coat protein of the bacteriophage. Advantageously, the coat protein is the pVIII coat protein. According to a preferred embodiment, the bacteriophage is a filamentous bacteriophage mutant, preferably a M13, fd or f1 mutant.

The native sequence of the pVIII coat protein of M13 mutant is the following: A**EGD**DPAKAAFDSLQASATEYIGYAWAMVVVIVGATIGIKLFKKFTSKAS (SEQ ID NO:19).

In an embodiment of the invention, the three native amino acid EGD (in positions 1, 2 and 3 in SEQ ID NO:19) are replaced by said amino acid sequence of 4 to 17 amino acids, preferably 5 to 12 amino acids, more preferably 8 amino acids, according to the invention. Consequently, the phage displays a pVIII protein wherein the three native amino acid EGD are replaced by an amino acid sequence of 4 to 17 amino acids, preferably 5 to 12 amino acids, more preferably 8 amino acids.

According to an embodiment of the invention, at least one amino acid sequence is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17 and SEQ ID NO:18.

Accordingly, the invention also relates to bacteriophages comprising a modified pVIII coat protein, wherein said modified pVIII coat protein has an amino acid sequence selected from the group consisting of SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36 and SEQ ID NO:37. Typically, these bacteriophages are filamentous bacteriophages. Particularly, these bacteriophages are selected from M13, fd and f1 mutants.

The bacteriophage according to the invention may further comprise genetically modified pIII and/or pIX coat proteins, capable to selectively recognize targets, such as for example pathogens, small molecules, proteins, viruses, ions, micro-organisms or the like.

Typically, the bacteriophages according to the invention are genetically engineered.

In one embodiment, the bacteriophages according to the invention are biotinylated.

The invention also relates to nucleic acid sequences encoding an amino acid sequence selected from the group consisting of SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36 and SEQ ID NO:37.

It is another object of the invention to provide a biosensor comprising a bacteriophage according to the invention. According to an embodiment, the biosensor further comprises a substrate at least partially coated with at least one metal oxide. Preferably, the metal oxide is Al_2O_3, Ta_2O_5 or TiO_2.

This invention also relates to a method of detecting and/or quantifying targets in a sample, comprising the following steps:

- contacting a sample with a bacteriophage according to the invention, said bacteriophage being or not being immobilized on a metal oxide surface,
- in the event that it was not immobilized on a metal oxide surface, further immobilizing the bacteriophage on a metal oxide surface;
- then detecting and/or quantifying the complex formed by interaction of said target and said bacteriophage.

[DETAILED DESCRIPTION OF THE INVENTION]

As described above, the invention provides bacteriophages wherein a coat protein of said bacteriophage is modified with at least one amino acid sequence, said amino acid sequence comprising 4 to 17 amino acids, the modified coat protein being capable of binding to a metal oxide surface.

Without being linked to any theory, the Applicant thinks that the presence of negatively charged amino acid, i.e. either alanine (A), asparagine (D) or glutamine (E), next to the ends of the inserted amino acid sequence may have some influence in the selective and/or specific binding to the metal oxide surface.

Preferably, the amino acid sequence is inserted between a residue (A) and a residue (D) or (E), of the native coat protein.

It is furthermore preferred that the amino acid sequence has a positively charged, basic amino acid, i.e. either lysine (K) or arginine (R), at about the middle of the sequence. Without wanting to be bound to any theory, it is believed that the presence of a positively charged, basic amino acid at about the middle of the amino acid sequence causes the peptide to kink at about the middle due to electrostatic repulsion and thus enhances the direction of the terminal acidic, negatively charged amino acid or acids to the metal oxide surface.

According to one embodiment, the amino acid sequence comprises from 4 to 17, preferably from 5 to 12, preferably from 6 to 10, more preferably from 7 to 9, and even more preferably about 8 amino acids. Very good results were obtained when using an amino acid sequence with 8 amino acids. The most preferred peptides

present a positively charged, basic amino acid, such as for example lysine (K) or arginine (R) in the middle of the sequence, typically on the 4^{th} or 5^{th} position when the amino acid sequence is of 8 amino acids. As set forth above, it is believed that the presence of a positively charged, basic amino acid in middle positions may cause the peptide to kink in the middle due to electrostatic repulsion and thus enhances the direction to the metal oxide surface of the acidic, negatively charged amino acids, bordering to the sequence, i.e. present just before and just after the 4-17 amino acid sequence. Moreover, the presence and number of polar, non-charged groups (especially S, T, Q, N) and non-polar ones (especially A, V, P) may be used to further refine adsorption behavior.

Suitable metallic oxides include, but are not limited to, binary or ternary oxides of Al, Ta, Ti, Zn, Sn, Nb, Zr, Sr, W, Ba. Preferably, for integrated biosensor applications, the metal oxide is selected from the group consisting of the valve metals, such as for example Al, Ta, Ti, Zr, W, more preferably Al_2O_3, Ta_2O_5, and/or TiO_2. The metal oxide may be amorphous or under crystalline form.

In a biological medium these metal oxide surfaces may become protonated, presenting a positive surface charge density in the biological medium. For example, for Al_2O_3, according to published values for the point of zero charge (PZC) of Al_2O_3 (PZC $\cong$ 9) [12], it is to be expected that at a neutral pH, the alumina surface becomes protonated. As a result, the specific affinity of the amino acid sequences with metal oxide surfaces, according to the invention, seems to be governed by both structural features and electrostatic interactions.

Suitable bacteriophages include, but are not limited to, filamentous bacteriophages, for example of the inoviridae family, such as for example M13 or fd or f1 mutants. The bacteriophage mutant may also comprise a further genetic modification on the same or another coat protein so as to render it capable of selectively recognizing *in-situ* specific targets in solution or suspension. Preferably, a further genetic modification occurs on the pIII or pIX coat protein.

According to an embodiment of the invention, the bacteriophages may be immobilized on a metal oxide surface, due to the affinity of the modified PVIII coat protein thereto. This immobilization may either be an *ex-situ* immobilization,

i.e. before contact of the bacteriophage with a target biological solution, or an *in-situ* immobilization of the bacteriophage contained in a biological medium by using the diagnostic event itself, i.e. capturing of the target by the phage, followed by phage immobilization on the metal oxide surface.

The invention also relates to nucleic acid sequence encoding an amino acid sequence selected from the group consisting of SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36 and SEQ ID NO:37.

The term "nucleic acid sequence" as used in this document includes genomic DNA, cDNA, synthetic DNA, and RNA. Preferably it means DNA, more preferably cDNA sequence coding for a fragment according to the invention. The nucleotide sequence may be of genomic or synthetic or recombinant origin, which may be double-stranded or single-stranded whether representing the sense or anti-sense strand.

The invention also relates to bacteriophages comprising a nucleic acid according to the invention.

The invention also relates to biosensors comprising a bacteriophage according to the invention. According to an embodiment, the biosensor further comprises a substrate at least partially coated with a metal oxide. Preferably, the metal oxide is Al_2O_3, Ta_2O_5 or TiO_2. Preferably, the substrate is at least partially coated with a thin dense Al_2O_3, Ta_2O_5 or TiO_2 film, having preferably a thickness of 50-1000 nm, preferably about 100 nm.

According to an embodiment, the biosensor includes a bacteriophage according to the invention, preferably M13, having a coat protein, preferably pVIII, modified with at least one amino acid sequence selected among SEQ ID No.1 to SEQ ID No.16 and a substrate at least partially coated with Al_2O_3. Typically, the biosensor includes a bacteriophage comprising a pVIII coat protein having an amino acid sequence selected from the group consisting of SEQ ID NO:20, SEQ ID NO:21,

SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34 and SEQ ID NO:35.

According to another embodiment, the biosensor includes a bacteriophage according to the invention, preferably M13, having a coat protein, preferably pVIII, modified with at least one amino acid sequence selected among SEQ ID No.17 to SEQ ID No.18 and a substrate at least partially coated with Ta_2O_5. Typically, the biosensor includes a bacteriophage comprising a pVIII coat protein of SED ID NO:36 or SEQ ID NO:37.

In a preferred embodiment, the bacteriophage mutant is a M13 mutant. Preferably, it also comprises a genetic modification on the pIII and/or pIX coat protein of M13, so that it can be used to selectively detect in-situ specific molecules in solution. The biosensor according to the invention may further comprise a substrate said substrate comprising a metal oxide surface.

The substrate may be any suitable conventional substrate which comprises a metal oxide surface. One example of a suitable substrate is a silicon wafer. The metal oxide surface may cover the surface of the substrate totally or partially. In a preferred embodiment the metal oxide surface covers the surface of the substrate only partially. It may for example be applied to predetermined areas of the substrate only. As the biosensor of the invention selectively and specifically to the metal oxide surface, parasitic effects during transducing and read-out are greatly reduced.

The metal oxide surface may be obtained by any suitable method known in the art, such as anodic oxidization.

According to an embodiment, the invention also relates to an array including a bacteriophage according to the invention.

According to an embodiment, the invention also relates to a kit including a bacteriophage according to the invention.

This invention also relates to a method of detecting and/or quantifying targets in a sample, comprising the following steps:

- contacting a sample with a bacteriophage according to the invention, said bacteriophage preferably being mutants of the inoviridae family, such as M13 or fd or f1 mutants, and preferably comprising a further genetic modification on the same or another coat protein, preferably on the pIII or pIX coat protein, so as to render it capable of selectively recognizing specific targets, such as pathogens, microorganisms or small proteins or the like, in solution or suspension

- simultaneously or sequentially, contacting the bacteriophage to a metal oxide surface in order to have the bacteriophage interacted with the metal oxide surface

- then detecting the complex formed by interaction of said target and said bacteriophage.

According to an embodiment, the phage may be first bound (or immobilized) on the metal oxide surface of the substrate and then contacted with the target-containing solution or suspension.

Alternatively, the phage is first contacted with the target-containing solution or suspension and then bound on the metal oxide surface of the substrate.

As used herein, the term

- "sample" refers to any sample containing material, such as food matrix, biological fluid (serum, urine...), biopsy material, environmental sample such as water as sewage water, freshwater, marine coastal water, ground water for example;
- "microorganisms" refers to any viable organism of microscopic size, including bacteria, cyanobacteria, chlamydiae, fungi, algae, protozoa and viruses;
- "pathogen" refers to any infectious agent, or germ, susceptible to cause disease or illness to a living being, including human; small molecules means small proteins of at most 100 amino acids, analytes having a molecular weight below 100 kDa;

- "target" refers to microorganisms, pathogens, small molecules, viruses, ions, proteins or the like;
- "binding", "to bind" (or any form of the verb to bind) refers to any detectable selective and/or specific affinity, and may apply to the specific affinity of the amino acid sequences to metal oxide surfaces, and/or to any recognition of a target by the phage. The affinity of the amino acid sequences to the metal oxide surface may result in immobilization of the bacteriophage according to the invention;
- "genetically engineered" and "genetically modified" are used herein interchangeably;
- A bacteriophage comprising "a coat protein" refers either to a bacteriophage comprising a single copy of said coat protein or comprising several copies (2, 5, 10, 100, 1000, 10 000, etc.) copies of said coat protein.

The present invention will be better understood with reference to the following examples and figure 1. These examples are intended to representative of specific embodiments of the invention, and are not intended as limiting the scope of the invention.

[FIGURE]

Fig. 1: ELISA analysis of the affinity of a bacteriophage having pVIII modified with SEQ ID NO:3, previously purified and biotinylated, for an Al_2O_3 surface.

[EXAMPLES]

EXAMPLE 1: Phage binding to an anodic Al_2O_3 surface using phage display technique [7]

Anodic Al_2O_3 surfaces were obtained by anodizing 400nm thick pure aluminium thin films, evaporated onto oxidised 3" silicon wafers. Thin film anodizing was carried out galvanostatically in 0.01 M ammonium octahydrate

pentaborate at 6 mA/cm^2, according to the procedures described by Vanovermeere et al. [8], leading to a 100 nm thick dense Al_2O_3 film.

As to the selection of amino acid sequences, a combinatorial library of random peptides was used, containing 8 amino acids inserted in the pVIII coat protein of M13 bacteriophages in place of native EGD.

Before starting the bio-panning protocol, which has already been described in detail by Soumillion [9], alumina surfaces were washed three times with 1 ml of Tris-HCl buffered saline (TBS). A round of selection consisted of incubating an equal amount of phages (1.5 1012) in a TBST buffer (0.14 M NaCl, 0.1M Tris-HCl, 0.1% v/v of Tween 20, pH = 7.25) for 1 hour. The non-bound phages were then collected, and the alumina surfaces were washed 10 times with TBST. An elution solution (0.2 M glycine-HCl/ 1g/l BSA, pH 2.2) was then added and incubated for 8 min in order to recover bound phages, followed by neutralisation in 1M Tris-HCl at pH 9. This elution procedure was repeated twice. Eluted phages were then amplified in E. coli for the next round of biopanning. A total of 6 biopanning rounds have been performed independently on two anodic Al_2O_3 surfaces. After the 6th round, individual phage clones were sampled randomly from each surface to identify peptide sequences.
During successful biopanning experiments, phage binding yield should increase from one round to another round of selection enrichment [10]. Significant enrichment was indeed observed in our experiments on anodic Al_2O_3 during the first 3 biopanning rounds. During the next 3 biopanning rounds, the yield of phage binding stabilised, resulting in a final 400-fold increase. These results, both the increase in the initial stage and the stabilisation in the later stage, are already indicative for significant peptide affinity towards anodic alumina.

In order to identify the peptide sequences with specific affinity towards anodic alumina, DNA analysis has been carried after the sixth round. Peptide sequences of selectants randomly isolated from anodic Al_2O_3 surfaces are shown

in Table 1. From the entire phage clone sample population, some very distinct features and consensus can be distinguished in the amino acid sequence, which is another indication of successful selection and of the specific affinity of some peptide sequences for anodic alumina surfaces.

The 6 rounds of panning procedures carried out on anodic alumina have allowed to isolate several phages that display peptides having identical sequences, like E-N-T-P-R-G-V-Q (7x), D-P-S-K-P-G-S-S (4x) and D-V-T-K-A-G-A-Q (2x). These selectant phage clones are grouped together in Table 1. To corroborate that these selectants indeed selectively bind to anodic Al_2O_3, 10^{12} of each of the 3 isolated phages were grown up and the ability of the axemic phage lots to selectively bind to anodic Al_2O_3 was verified by comparing to blanc experiments without any solid surface in solution. Ratio's in excess of 400 between the Al_2O_3 and blanc protocols were always obtained.

	SEQ ID is inserted after native	**Table 1.** Amino acid sequences for successfully analysed selectant phage clones.								SEQ ID is followed by native	
SEQ ID No. 1	A	D	P	A	A	R	T	Q	V	D	P
SEQ ID No. 2	A	E	N	T	P	R	G	V	Q	D	P
SEQ ID No. 3	A	D	P	S	K	P	G	S	S	D	P
SEQ ID No. 4	A	D	P	A	K	S	P	S	S	D	P
SEQ ID No. 5	A	D	V	T	K	A	G	A	Q	D	P
SEQ ID No. 6	A	D	P	G	P	K	P	S	T	D	P
SEQ ID No. 7	A	D	P	S	L	R	N	Q	T	D	P
SEQ ID No. 8	A	D	T	T	K	V	P	S	Q	D	P
SEQ ID No. 9	A	D	V	T	K	A	T	A	Q	D	P
SEQ ID No. 10	A	E	P	G	K	A	S	G	S	D	P
SEQ ID No. 11	A	E	P	G	K	G	S	A	M	D	P
SEQ ID No. 12	A	E	P	I	K	G	G	G	S	D	P
SEQ ID No. 13	A	E	P	P	S	G	L	L	R	D	P
SEQ ID No. 14	A	E	P	P	S	K	A	A	M	D	P
SEQ ID No. 15	A	E	P	S	K	A	A	G	T	D	P
SEQ ID No. 16	A	E	P	V	Q	K	A	G	S	D	P

EXAMPLE 2: Phage binding to a Ta_2O_5 surface using phage display technique [7]

Anodic Ta_2O_5 surfaces were obtained by anodizing pure Ta thin films. A library of random peptides was used, containing 8 amino acids inserted in the pVIII coat protein of M13 bacteriophages in place of native EGD was screened using the biopanning protocol of Example 1.

The sequences shown in table 2 were shown to exhibit selective and specific affinity to a Ta_2O_5 surface.

Table 2. Amino acid sequences for successfully analysed selectant phage clones (Ta_2O_5).

SEQ ID No. 17	D	P	A	K	G	P	A	T
SEQ ID No. 18	D	P	A	K	S	L	G	T

EXAMPLE 3: Biotinylation of the bacteriophages according to the invention does not prevent their binding to metal oxide surfaces

a) Bacteriophages of Example 1 modified with SEQ ID NO:3 have been purified and biotinylated (see protocols hereinafter). Their affinity for an Al_2O_3 surface has than been measured.

Phage purification by cesium chloride centrifugation

Equipment and reagents

L8-M Beckman ultracentrifuge

Swinging bucket SW40 rotor

Cesium chloride (Sigma)

TBS

Purified phage preparation

Method

- Dilute the phage suspension with TBS to reach a final volume of 12 ml
- Add 5,4 g of cesium chloride and mix to dissolve
- Centrifuge the solution in a SW40 rotor for 17 h and 200000 g at 15°C
- Collect the fraction containing the phages
- Dialyse the harvested solutions against TBS overnight in a float A Lyzer G2

Phage modification with NHS-biotin

Purified phage preparation

PEG 20%

Centrifuge Beckman, microfuge 18

NaCl 100 mM/$NaHCO_3$ 100mM buffer pH8

NHS-Biotin (Thermo scientific)

TBS

Method

- Add 1/5 volume of PEG in the 2 ml phage suspension
- Incubate for 1 h on ice
- Centrifuge 10 min at 10000 rpm (Centrifuge Beckman, microfuge 18)
- Discard the supernatant and resuspend the pellet in 1,2 ml of buffer (NaCl 100 mM/$NaHCO_3$ 100 mM pH 8)
- Divide the volume in 6 aliquots of 200 µl
- Prepare samples of NHS-biotin at different final concentration (0,01 mM, 0,1 mM, 1 mM and 10 mM)
- Add 2 µl of NHS-biotin in each sample in order to obtain different final concentration (0 mM, 0,1 µM, 1µM, 10 µM and 100 µm)
- Incubate for 1h30 at room temperature
- Add 1/5 volume of PEG and incubate for 15 min on ice
- Centrifuge 10 min at 10000 rpm (Centrifuge Beckman, microfuge 18)
- Repeat the two last steps 3 times

Resuspend the pellet in 200 µl of TBS

Adsorption on Al_2O_3 surface is then measured as described in Example 1. Results are presented in the table hereinafter:

NHS-biotin concentration	% of adsorbed phages when Al_2O_3 surface is present (10^{-2}% /cm²)	Blank: % of adsorbed phages without Al_2O_3 surface (10^{-2}%)	Ratio "% of adsorbed phages when Al_2O_3 surface is present" / blank
0%	0.169	0.00003	5634
0.01%	0.177		5892
0.10%	0.227		7556
1%	0.211		7024
10%	0.673		22429

b) Biotinylated bacteriophages prepared as described in a) have been tested for their ability to recognize streptavidine (ELISA protocol hereinafter).

ELISA

Equipment and reagents

24 wells plate (Nunclon Surface)

96 wells plate (VWR)

2% MPBS

TBS + 0,5% TWEEN 20

Streptavidin-Peroxidase Polymer, Ultrasensitive (Sigma)

3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system (Sigma T-8665)

H_2SO_4 1M

Phage preparation

Method

- Place the Al_2O_3 surface in the wells of the 24 wells plate
- Wash the wells 10 times with 1 ml TBS 1X + 0,5% TWEEN 20
- Add 50 µl of the phage suspension and 450 µl TBS 1X + 0,5% TWEEN 20 to the wells

- Incubate for 1h at room temperature under shaking
- Discard the solution and wash the wells 5 times with 1 ml de TBS 1X + 0,5% TWEEN 20 under shaking, 3 times with 1 ml PBS 1X + 0,05% TWEEN 20 and 3 times with1ml PBS 1X
- Add 400 µl/well HRP-streptavidin diluted 1 : 200 in 2% MPBS
- Incubate for 1h at room temperature
- Discard the solution and wash wells 3 times with TBS 1X + 0,5% TWEEN 20 and 3 times with PBS 1x
- Add 400 µl TMB system solution to each well and incubate at room temperature for 10 min in the dark.
- Quench the reaction by adding 400 µl H_2SO_4 1M
- Read the plate at 450 nm

Results (see also Fig. 1)

Sample	NHS-biotin concentration (µM)	Phage suspension	Al_2O_3 surface	HRP-streptavidin 1 : 200	Absorbance
1	0	+	+	+	0,176
2	0,1	+	+	+	0,319
3	1	+	+	+	0,596
4	10	+	+	+	0,901
5	100	+	+	+	1,341
6	0	+	-	+	0,091
7	1	+	-	+	0,226
8	10	+	-	+	0,189
9	100	+	-	+	0,485
10	0	-	+	+	0,212
11	0	+	+	-	0,050
12	100	+	+	-	0,054

As shown in figure 1, for a same concentration in streptavidin, when the concentration in biotin is increased, the recognition is not lost and signal is proportionally increased (col 1-5). Without alumina (col.6-9), there is no recognition. Col. 10-12 are controls.

References

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CLAIMS

1. A bacteriophage comprising a coat protein modified with at least one amino acid sequence, wherein said amino acid sequence comprises 4 to 17 amino acids, and wherein the modified coat protein is capable of binding to a metal oxide surface.

2. The bacteriophage according to claim 1, wherein said amino acid sequence comprises 5 to 12 amino acids, preferably about 8 amino acids.

3. The bacteriophage according to claim 1 or 2, wherein said coat protein is the pVIII coat protein and the bacteriophage is a filamentous bacteriophage.

4. The bacteriophage according to any one of claims 1 to 3, wherein said bacteriophage is a M13, a fd or a f1 mutant.

5. The bacteriophage according to any one of claims 1 to 4, wherein said amino acid sequence is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17 and SEQ ID NO:18.

6. The bacteriophage according to any one of claims 1-5, wherein said coat protein is the pVIII coat protein, and wherein said amino acid sequence is inserted in place of the amino acids EGD which are present in positions 2,3,4 of the amino acid sequence of the native pVIII coat protein, said amino acid sequence of the native pVIII coat protein being as shown in SEQ ID NO:19.

7. The bacteriophage according to any one of claims 1 to 6, wherein said bacteriophage further comprises a modified pIII coat protein and/or a modified pIX coat protein, capable to bind to targets.

8. A bacteriophage comprising a modified pVIII coat protein, wherein said modified pVIII coat protein has an amino acid sequence

selected from the group consisting of SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36 and SEQ ID NO:37.

9. The bacteriophage of claim 8, wherein said bacteriophage is a filamentous bacteriophage.

10. The bacteriophage of claim 9, wherein said bacteriophage is a M13, a fd or a f1 mutant.

11. The bacteriophage according to any one of claims 8-10, wherein said bacteriophage further comprises a modified pIII coat protein and/or a modified pIX coat protein.

12. The bacteriophage according to any one of claims 1-11, wherein said bacteriophage is genetically engineered.

13. The bacteriophage according to any one of claims 1-12, wherein said bacteriophage is biotinylated.

14. A nucleic acid sequence encoding an amino acid sequence selected from the group consisting of SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36 and SEQ ID NO:37.

15. Biosensor comprising a bacteriophage as defined in anyone of claims 1 to 13.

16. Biosensor according to claim 15, further comprising a substrate at least partially coated with at least one metal oxide.

17. Biosensor according to claim 16, wherein the metal oxide is Al_2O_3, Ta_2O_5 and/or TiO_2.

18. Method for detecting and/or quantifying targets in a sample, comprising the following steps:

- contacting a sample with a bacteriophage as defined in anyone of claims 1 to 13;
- said bacteriophage being or not being immobilized on a metal oxide surface,
- in the event that it was not immobilized on a metal oxide surface, further immobilizing the bacteriophage on a metal oxide surface;
- then detecting and/or quantifying the complex formed by interaction of said target and said bacteriophage.

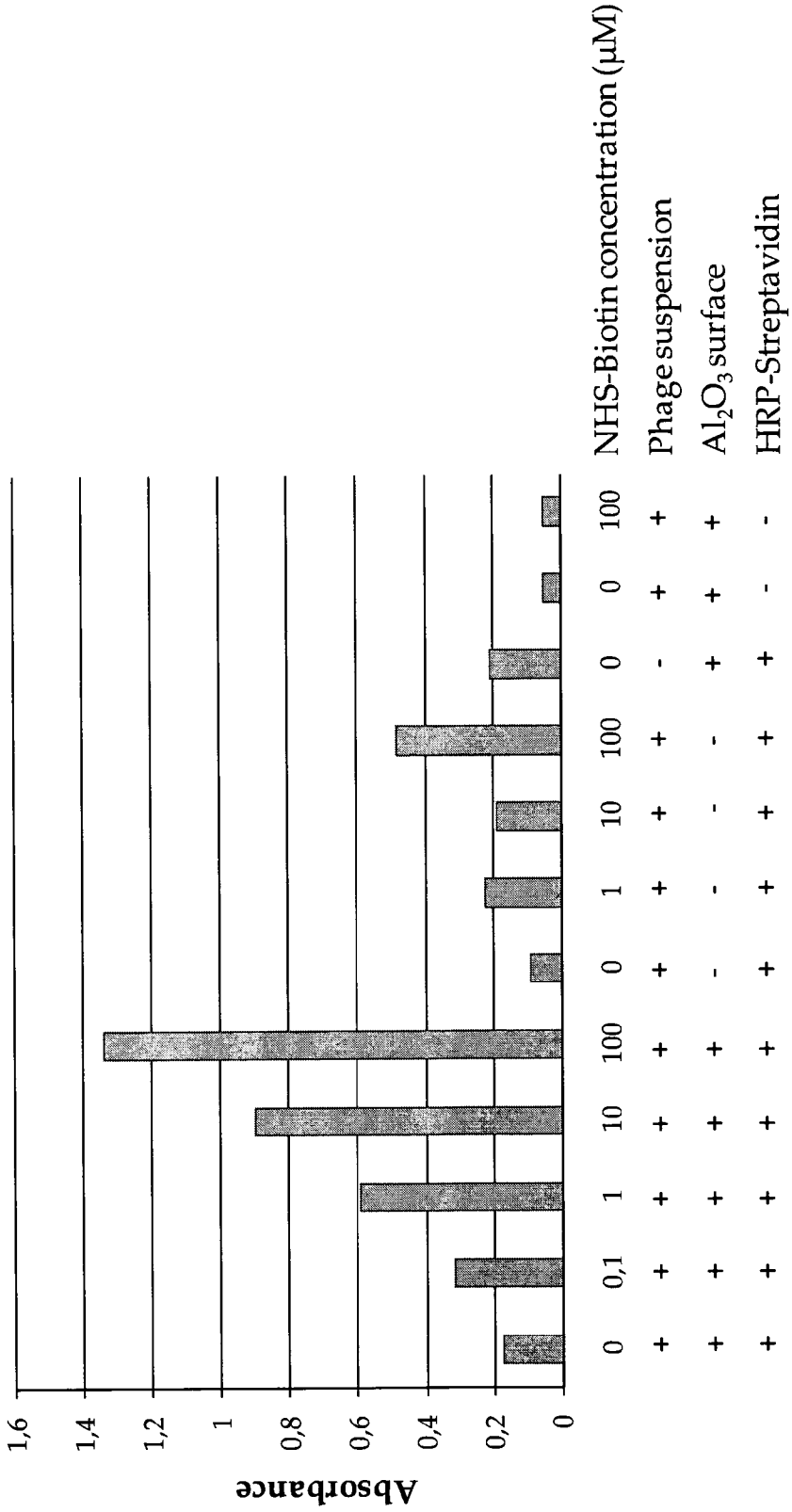
Absorbance
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
NHS-Biotin concentration (µM)
Phage suspension
Al_2O_3 surface
HRP-Streptavidin
0 0,1 1 10 100 0 1 10 100 0 0 100
+ + + + + + + + + + + +
+ + + + + - - - - + + +
+ + + + + + + + + + + -

FIG. 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/057279

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N7/00 C12N15/10
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BROWN S: "Engineered iron oxide-adhesion mutants oof the Escherichia coli phage lambda receptor" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES (PNAS), NATIONAL ACADEMY OF SCIENCE, US LNKD-DOI:10.1073/PNAS.89.18.8651, vol. 89, 1 September 1992 (1992-09-01), pages 8651-8655, XP002240665 ISSN: 0027-8424 * abstract page 8651, right-hand column, paragraph 1 page 8655, left-hand column, paragraph 2; figure 3 ----- -/--	1-18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 August 2010	19/08/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Herrmann, Klaus

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/057279

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WHALEY S R ET AL: "SELECTION OF PEPTIDES WITH SEMICONDUCTOR BINDING SPECIFICITY FOR DIRECTED NANOCRYSTAL ASSEMBLY" NATURE, NATURE PUBLISHING GROUP, LONDON, GB LNKD- DOI:10.1038/35015043, vol. 405, 8 June 2000 (2000-06-08), pages 665-668, XP002909553 ISSN: 0028-0836 page 665, left-hand column, last paragraph - right-hand column, paragraph 1	1-18
A	LEE SOO-KWAN ET AL: "Cobalt ion mediated self-assembly of genetically engineered bacteriophage for biomimetic Co-Pt hybrid material" BIOMACROMOLECULES, vol. 7, no. 1, January 2006 (2006-01), pages 14-17, XP002594759 ISSN: 1525-7797 page 14, right-hand column, last paragraph page 15, left-hand column, last paragraph	1-18
A	KRIPLANI ET AL: "Selecting peptides for use in nanoscale materials using phage-displayed combinatorial peptide libraries" CURRENT OPINION IN BIOTECHNOLOGY, LONDON, GB LNKD- DOI:10.1016/J.COPBIO.2005.07.001, vol. 16, no. 4, 1 August 2005 (2005-08-01) , pages 470-475, XP005006174 ISSN: 0958-1669 figure 1 table 1	1-18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2010/057279

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-18(partially)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 1-18(partially)

Claim 1 covers all bacteriophages comprising a coat protein modified with at least one amino acid sequence, wherein said amino acid sequence comprises 4 to 17 amino acids, having the desired characteristic of binding to a metal oxide surface. However, the application provides support (Art. 6 PCT) and disclosure (Art. 5 PCT) for only a limited number of such bacteriophages. Besides the filamentous bacteriophage M13 comprising comprising coat protein pVIII modified by inserting an amino acid sequence according to SEQ ID NOs:1-18 in place of the amino acids EGD present in positions 2, 3 and 4 of the native pVIII coat protein, no other bacterionphages have been shown to have the desired characteristic. Consequently, the search has been limited to bacteriophages comprising said characteristics, their use and the nucleic acid sequences as defined in claim 14 (cf. Art. 17(2)(a)(ii) PCT).

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.2), should the problems which led to the Article 17(2) declaration be overcome.