Research Article

Journal of PeptideScience

Received: 15 July 2010 Revised: 12 September 2010 Accepted: 18 September 2010 Published online in Wiley Online Library: 30 November 2010

(wileyonlinelibrary.com) DOI 10.1002/psc.1315

Phages recognizing the Indium Nitride semiconductor surface via their peptides‡

Elias Estephan,[a] Marie-Belle Saab,[b] Marta Martin,[b] Christian Larroque,[c] Frédéric. J. G. Cuisinier,[a] Olivier Briot,[b] Sandra Ruffenach,[b] Matthieu Moret[b] and Csilla Gergely[b]*

Considerable advances in materials science are expected via the use of selected or designed peptides to recognize material, control their growth, or to assemble them into elaborate novel devices. Identifying specific peptides for a number of technologically useful materials has been the challenge of many research groups in recent years. This can be accomplished by using affinity-based bio-panning methods such as phage display technologies. In this work, a combinatorial library including billions of clones of genetically engineered M13 bacteriophage was used to select peptides that could recognize improved indium nitride (InN) semiconductor (SC) material. Several rounds of biopanning were necessary to select the phage with the higher affinity from the low variant library. The DNA of this specific phage was extracted and sequenced to set up the related specific adherent peptide. Atomic force microscopy (AFM) is used to demonstrate the real affinity of a selected phage for the InN surface. Due to the possibility of its functionalization with biomolecules and its important physical properties, InN is a promising candidate for developing affinity-based optical and electrical biosensors and/or for biomimetic applications. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: InN semiconductor; phage display; peptides; biomolecules; functionalization; atomic force microscopy

Introduction

The long history of semiconductors (SCs) has been largely devoted to the investigation of their growth mechanisms and their electrical, optical, and magnetic properties. This has led to sustained progress in the synthesis of materials of higher purity, to functionalized SCs with specifically tailored properties and to the development of technologies for the processing and assembly of SCs into functional structures and devices. Recent research on the interaction between organic molecules and inorganic materials provided the assembly of functional hybrid structures for nanoelectronics and high performance biosensors. While much remains to be accomplished, these fascinating materials clearly offer great potential for the development of revolutionary hybrid structures and devices, and work in this field has propelled SCs to a point that their entry into the realm of nanobiotechnology and nanomedicine is now a reality.

Among the SCs, nitrides containing gallium, aluminium, and their alloys including those with low indium content have been widely investigated in the last decades; except for indium nitride (InN), the least known of the nitride family. With a bandgap lying between 0.6 and 0.7 eV [1–3], InN alone or combined with other nitrides like GaN or AlN offers huge perspectives in a wide range of fields including optoelectronics, solar cells, and terahertz applications. This motivated a worldwide effort to achieve high quality material by using several growth techniques. Nowadays, although the crystalline quality of InN layers has been considerably improved, all InN samples exhibit an electron accumulation layer at their surface, reducing drastically the field of applications that are expected. Only one kind of application has benefited from this unusually high surface sensitivity: sensors [4] and biosensors [5]. Recently, it was demonstrated that InN samples grown by molecular beam epitaxy exhibit an enhanced concentration of hydrogen near the surface [6]. Similar results were obtained by the same group on InN samples grown by metal organic vapor phase epitaxy (MOVPE). However, although hydrogen is expected to behave like a donor in InN [7], no correlation between the electron and hydrogen concentrations, which are located near the surface region, has been established yet.

In order to be integrated with biological molecules or to be used in biological systems, InN has to accomplish biocompatibility and be preferably functionalized to assure recognition of biomolecules. To reach biocompatibility, InN has to be functionalized with linkers [8], reducing toxicity and accomplishing materials' stability. In general, coupling proteins to the surface of materials has been carried out via silanization or by using linkers such as thiols or phosphane groups [9–11]. Nowadays new methods, which allow the generation of short proteins and peptides that

* *Correspondence to: Csilla Gergely, Groupe d'Etude des Semi-conducteurs, UMR 5650, CNRS-Université Montpellier II, 34095, Montpellier Cedex 5, France. E-mail: Gergely@ges.univ-montp2.fr*

a *EA 4203, UFR Odontologie, Université Montpellier I, 34193 Montpellier Cedex 5, France*

b *Groupe d'Etude des Semi-conducteurs, UMR 5650, CNRS-Université Montpellier II, 34095, Montpellier Cedex 5, France*

c *IRCM/INSERM896, Centre Régional de Lutte contre le Cancer Val d'Aurelle-Paul Lamarque, Université Montpellier I, 34298 Montpellier, France*

‡ *Special issue devoted to contributions presented at the E-MRS Symposium C "Peptide-based materials: from nanostructures to applications", 7-11 June 2010, Strasbourg, France.*

can specifically recognize different materials and grow inorganic building blocks, are appearing. Selected peptides can play the role of the previously used common linkers and might bypass and exceed their recognition limits.

To identify the appropriate compatibilities and combinations of biological inorganic materials, phage display technology by means of a combinatorial library of the genetically engineered M13 bacteriophage can be used to select peptides, revealing specific recognition for the inorganic SC material. Phage display consists of successive rounds of incubating, rinsing, elution, and amplifying steps to finally isolate phages with the best affinities toward the target. Selection of inorganic binding peptides through display technologies was largely previously used by us for GaAs and InP III–V class SCs; ZnS, ZnSe, CdSe, and CdS II–VI class SCs; and by others for magnetic materials including FePt, CoPt, and Co [12–18]. Also, this technology was applied on biomaterials like the chlorine-doped polypyrrole [19]. Some of these peptides or phages have been used to assemble inorganic particles [12–14,20], as molecular building blocks to nucleate and arrange quantum dots [13], template SC nanowires [14,15], and for producing multidimensional liquid crystals and films [13]. Moreover, these peptides have been used as linkers to couple biomolecules to a new range of sensing devices in order to ameliorate the detection threshold [21]. Other genetically engineered peptides have been employed for the formation (biofabrication, synthesizing, and controlling the nucleation and growth) of the selected compounds [22–25].

In this work, phage display technology was explored for the InN SC as target substrate. After three rounds of biopanning, several phages were recovered and then their DNA was sequenced to finally set up the sequence of the peptide that presents affinities for the InN surface. A specific phage was chosen and its affinity toward InN was studied using atomic force microscopy (AFM).

Materials and Methods

Substrate Growth

The InN layer was grown by MOVPE [26], using trimethylindium and ammonia as group III and group V precursors respectively. The growth was performed at 450 °C under a nitrogen atmosphere, by using group V/III molar ratio of 15 000. About 300 nm InN was grown subsequently to a 1-μm GaN layer onto sapphire in the same growth run. The resulting In-polar epilayer presents a root-mean-square (RMS) roughness of 12 nm measured on a 10×10 μm AFM image.

Substrate Preparation before Functionalization

The InN samples were carefully cleaned by successive treatments with trichloroethane, acetone, and methanol, rinsed in deionized water and then dried with nitrogen. In order to remove native oxide, the samples were etched with dilute HCl (HCl/H_2O : 1/10) for 30 s and then immersed in deionized water and dried with nitrogen. The morphology of the bare sample surface was monitored by AFM (not shown).

Screening Phage Display Libraries

For our investigation, we used the 'Ph.D.TM-12 Phage Display Peptide Library Kit', supplied by New England Biolabs (Beverly, MA, USA). This M13 bacteriophage library contains 10^9 different 12-mer peptides present at the *N*-terminal of the minor protein PIII. The library was used without any previous amplification, as recommended by the supplier. Prior to the phage display, the InN surfaces were preliminarily prepared by appropriate chemical etching; thus, native oxides have been removed assuring the specific phage to reach the SC. The bacteriophage library was then exposed to the SC surface in phosphate-buffered saline solution containing 0.1% Tween-20 (PBST). These conditions were necessary to reduce unspecific interaction on the surface [5]. Following rocking for 1 h at room temperature, the surfaces were washed 5–10 times using PBST to rinse the unbound phages. Bound phages were eluted from the surface by the addition of glycine–HCl (pH 2.2) for 10 min, then transferred to a fresh tube, and neutralized with Tris–HCL (pH 9.1). The chosen pH conditions are intrinsic to the phage display method: the selection of the peptide is based on the elution condition (pH 2.2), namely on desorption propensity. The eluted phages were titered and amplified by infecting *Escherichia coli* ER2738 host bacterial cells. Three rounds of biopanning were performed and several phages were recovered, amplified, and sequenced. Great care was given to the accuracy of experiments; thus, after each round the target sample was changed with a new one of the same type (from the same wafer) to retain a similar surface condition at the exposure of the sample. Also, before each elution step the sample was moved to another well in order to not elute the phages sticking to the plastic.

Surface Functionalization

Once the sequences were determined by phage display, the appropriate phage which presented the most polar peptide was used for the functionalization. After the steps of cleaning and deoxidation, the 5×5 mm samples were incubated in a solution of the chosen phages for 2 h. The phage's solution can be prepared using the 2 × TY medium (a mixture of 16 g tryptone, 10-g yeast extract, and 5-g NaCl in 1 l of H_2O) or PBST. Phage adsorption was followed by a thorough rinsing step with PBST to remove unbound and excess phages.

Surface Topography Imaging by AFM

AFM images of the functionalized InN were recorded in PBST buffer, with an Asylum MFP-3D head equipped with a Molecular Force Probe 3D controller (Asylum Research, Santa Barbara, CA, USA). The selected imaging mode was the tapping (AC) mode and rectangular silicon nitride cantilevers from Olympus (OMCL-RC800PSA, Tokyo, Japan) were used at a drive frequency of 18 kHz. Height and phase images were recorded but in this paper we show only the height images. We always performed several scans over a given surface area assuring reproducible images. Typically, the images were taken at 1 Hz scan rate and digitized in 512×512 pixels. They were first recorded at $10 \times 10\ \mu m^2$ scan size, then $5 \times 5\ \mu m^2$, and finally at $1 \times 1\ \mu m^2$ for a better resolution.

Results and Discussions

Biofunctionalization of the InN substrate was achieved by means of phages expressing peptides with specific binding. The phages and then the related peptides were identified using the phage display method. Normally, five fundamental steps are required for better selection affinity: (i) the dilution of the original commercial

Journal of PeptideScience

Table 1. Sequences of the peptides expressed by the selected phages

Selected peptides (%)	MW[a]	pI[b]	charge	II[c]	AI[d]	H[e]
Group 1						
STLMTTTYHSVS (8.3)	1326.61	6.46	0	17.05	56.67	0.017
QGAHYEYSRTEL (8.3)	1452.66	5.40	−2, +1	57.45	40.83	−1.425
IPGDAGKGLHMT (8.3)	1195.6	6.74	−1, +1	−9.78	73.33	−0.175
YDTTSSPPRLTR (8.3)	1392.7	8.75	−1, +2	78.81	32.5	−1.408
GMIKAAHERPLR (8.3)	1377.77	10.84	−1, +3	27.12	81.67	−0.65
VLSNSLPTAIST (8.3)	1201.66	5.49	0	25.22	130	0.767
Group 2						
GMATEATVHELA (8.3)	1228.58	4.51	−2	35.50	81.67	0.275
LIRGLLIGFGRN (8.3)	1327.81	12	+2	12.38	162.5	0.792
YPLLPESPTDAN (16.6)	1315.63	3.67	−2	102.65	73.33	−0.725
NWSAEKAKLYDS (8.3)	1410.68	6.07	−2, +2	29.23	49.17	−1.225
QNSHRVALENNT (8.3)	1381.67	6.75	+1, −1	2.09	65	−1.408

[a] MW is the theoretical monoisotopic molecular masses.
[b] pI is the isoelectric point.
[c] II is the instability index of a peptide: II <40 predicts as stable, II >40 predicts an unstable peptide.
[d] AI is the aliphatic index.
[e] H is the hydropathicity; it is calculated as the sum of hydropathy values Kyte *et al*. (1982) of all the amino acids divided by the number of residues in the sequence.
The above-mentioned values are calculated using computer programs on http://expasy.org.

library or the amplification of an eluted library, (ii) the incubation of the phage particles with the target, (iii) the rinsing step in which the non-specific binders can be removed, (iv) the elution step in which the phages and the target can occur in extreme conditions of pH, and (v) the repetition of the mentioned steps for purifying. Commonly, these steps are repeated two to four times; when this is accomplished, the phage population becomes less and less diverse as it becomes more and more enriched in the limited number of variants with binding capacity. The peptide that comes into contact with the target can be determined after sequencing the genome of the phages to finally determine the specific ligands to the target.

After three rounds of biopanning, 12 phages are recovered and amplified to finally extract their DNA and to set up the sequence of the related specific peptides. As a first step we have obtained only six phages, and the peptides expressed by these first six phages constitute the first group of sequences shown in Table 1. For comparison, several physico-chemical characteristics of the resulted peptides have been calculated using computing programs on http://expasy.org. The values have been included in Table 1 as follows: the molecular theoretical monoisotopic molecular mass (MW), the isoelectric point (pI), and the instability index of the peptide (II) (II <40 predicts a stable and II >40 predicts an unstable peptide). The aliphatic index (AI) has been also calculated as a measure of the relative volume occupied by the aliphatic side chain of the Ala, Val, Leu, and Ile amino acids. An increase in the AI increases the thermostability of the peptide. Hydropathicity (H) of the peptide is calculated as the sum of hydropathy index values [27] of all the amino acids divided by the number of residues in the sequence. The larger the number, the more hydrophobic is the amino acid or the peptide.

In order to monitor the functionalization of InN with the selected phages, AFM was employed. The phage expressing the QGAHYEYSRTEL sequence peptide was chosen, based on the abundance of the polar amino acids in its sequences [11].

Figure 1 shows high resolution AFM images of a bare InN surface before (Figure 1(a) and (c)) and after adsorption of phages (Figure 1(b) and (d)). The size of the surface features observed on the naked InN surface ranges from 100 to 150 nm, as depicted in Figure 1(c).

Comparison of the AFM figures recorded before and after adsorption of phages onto InN surface evidences a growth of these features to sizes between 200 and 300 nm, demonstrating adsorption of the phages. The roughness (calculated as RMS on $5 \times 5\,\mu m^2$ images) is RMS $= 14.0 \pm 0.5$ nm for both the bare and functionalized InN substrate, respectively. However, a closer look at higher resolution $1 \times 1\,\mu m^2$ images indicates a certain levelling of surface roughness when phages are adsorbed on InN as RMS decreases from 12.8 to 8.9 nm. The underlying features characteristic to the InN surface are still visible after adsorption, suggesting that the phages adsorb in an ordered manner onto InN (Figure 1(d)). The used M13 bacteriophages are filamentous viruses (composed of circular single stranded DNA) that are about 1 μm long and 5–6 nm in diameter. As expected, these phages can recognize the surface via their adhesion peptides (the p3 protein) found at the end of the phage particles known to adopt a rectangular form. A tentative model illustrating adsorption of phages and their ordered arrangement after the rinsing step is schematized in Figure 2. Phages usually adsorb in an 'end-on' configuration driven by the adhesion peptides located at one of its ends.

Our further objective was to identify the sequence of these adhesion peptides presenting a good affinity for InN. It is evident that surface modification via extremely small (<1 nm) peptides instead of the large phages could be a better solution for applications in microelectronics and biosensing too. As the first group of peptides presented in Table 1 were all different, a second step of extraction and sequencing was performed. The corresponding sequences of the peptides expressed by the selected phages are also shown in Table 1 and noted as group 2. A comparison based on the phsyico-chemical properties of the obtained peptides (shown in Table 1) are charged and stable as the

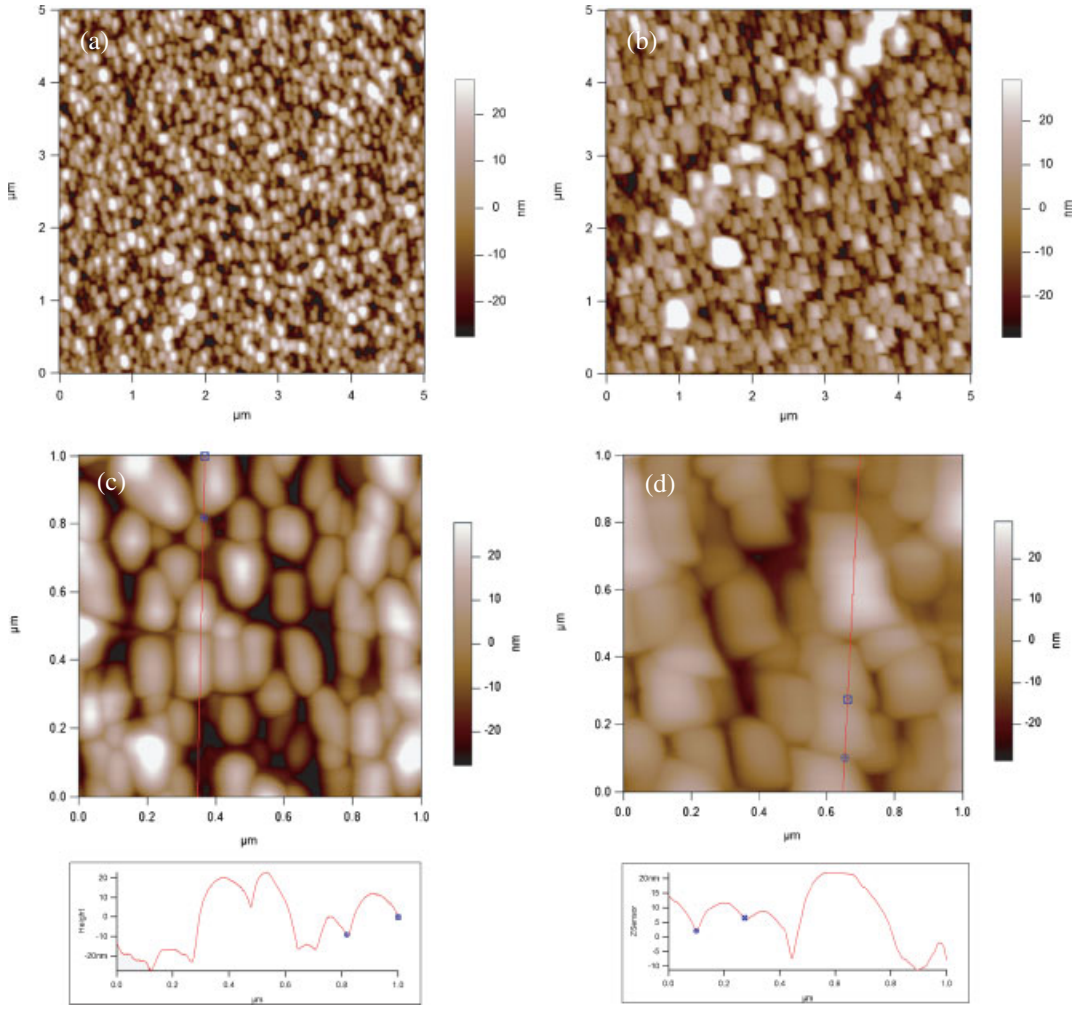

Figure 1. AFM image (a) 5 × 5 µm^2 scan on bare InN, (b) 5 × 5 µm^2 scan on functionalized InN, (c) 1 × 1 µm^2 scan on bare InN showing a section profile, (d) 1 × 1 µm^2 scan on functionalized InN showing a section profile.

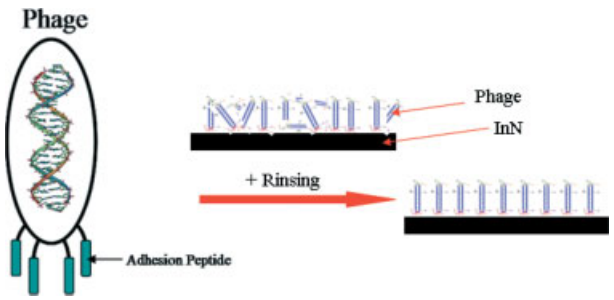

Figure 2. Schema depicting the functionalization procedure: the phages recognize the surface via their peptides.

instability index II is <40. Most of them have an isoelectric point lying in the domain of 5–7. Several similarities in the sequences can be noted, such as the GM123A4567L8 (the numbers denote any kind of amino acids) between the GMIKAAHERPLR peptide in the first group and the GMATEATVHELA peptide in the second group. These two peptides are predicted to be stable, very charged, and present the same AI. Other common sequences are also to be noted, such as the Y1TT between the first and the fourth peptide in the first group, the ELA between the first and the fifth in the second group, the S1S23T between the first and the sixth peptide, also in the first group, and these peptides are not charged. The statistically obtained apparition (expressed in percentage) of an identical peptide sequence was calculated. As the YPLLPESPTDAN appears two times over 12, this peptide can be expected to be a good binder. To the best of our knowledge, this is the first time that a specific peptide has been identified for the InN material.

Hence, our further studies will concern the use of this specific peptide for functionalization of InN. The binding forces between the selected YPLLPESPTDAN peptide and InN should be also investigated. However, we might already suggest that the positively charged donor states of the InN drives adsorption of this peptide through its two negative charges. Adsorption of small anions (Cl^-, OH^-) onto the positively charged surface states of InN has been previously reported [28], but never observed with larger molecules.

Conclusion

Due to its proven robust surface properties and an unusually strong surface electron accumulation, InN is an excellent candidate for molecular detection with promising biomedical applications. Our work reveals the outstanding benefits of the developed specific

biofunctionalization by means of phages or peptides: Phage display technology was elaborated on the InN SC and several peptides were sequenced. After the third round of biopanning, a specific phage was chosen and its adsorption was demonstrated via AFM imaging. When looking for the peptide with the highest affinity for the InN material, the YPLLPESPTDAN peptide has been selected based on its highest apparition frequency in the phage display. The sequenced phages and/or their related peptide can be used as linkers for the InN surface with promising applications in directed crystal growth. Combining biomolecules with InN might promote assembly of various materials into original functional devices with novel mechanical, electronic, photonic, or magnetic properties.

Acknowledgement

This work was supported by the COST – EU Action MP0702: 'Toward functional sub-wavelength photonic structures'.

References

1 Wu J, Walukiewicz W, Yu KM, Ager JW, Haller EE, Lu H, Schaff WJ, Saito Y, Nanishi Y. Unusual properties of the fundamental band gap of InN. *Appl. Phys. Lett.* 2002; **80**: 3967–3971.
2 Davydov VY, Klochikhin AA, Emtsev VV, Ivanov SV, Vekshin VV, Bechstedt F, Furthmuller J, Harima H, Mudryi AV, Hashimoto A, Yamamoto A, Aderhold J, Graul J, Haller EE. Band gap of hexagonal InN and InGaN alloys. *Phys. Status Solidi B* 2002; **230**: 787–795.
3 Hori M, Kano K, Yamaguchi T, Saito Y, Araki T, Nanishi Y, Teraguchi N, Suzuki A. Optical properties of InxGa1 – xN with entire alloy composition on InN buffer layer grown by RF-MBE. *Phys. Status Solidi B* 2002; **234**: 750–754.
4 Lu H, Schaff WJ, Eastman LF. Surface chemical modification of InN for sensor applications. *J. Appl. Phys.* 2004; **96**: 3577–3600.
5 Chen CF, Wu CL, Gwo S. Organosilane functionalization of InN surface. *Appl. Phys. Lett.* 2006; **89**: 252109–252113.
6 Darakchieva V, Lorenz K, Barradas NP, Alves E, Monemar B, Schubert M, Franco N, Hsiao CL, Chen LC, Schaff WJ, Tu LW, Yamaguchi T, Nanishi Y. Hydrogen in InN: a ubiquitous phenomenon in molecular beam epitaxy grown material. *Appl. Phys. Lett.* 2010; **96**: 81907–81911.
7 Limpijumnong S, Van de Walle CG. Passivation and doping due to hydrogen in III-nitrides. *Phys. Status Solidi B* 2001; **228**: 303–307.
8 Stutzmann M, Garrido JA, Eickhoff M, Brandt MS. Direct biofunctionalization of SCs : a survey. *Phys. Status Solidi A* 2006; **203**: 3424–3437.
9 Palestino G, Legros R, Agarwal V, Pérez E, Gergely C. Functionalization of nanostructured porous silicon microcavities for glucose oxidase detection. *Sens. Actuators, B* 2008; **135**: 27–34.
10 Palestino G, Agarwal V, Aulombard R, Pérez E, Gergely C. Biosensing and protein fluorescence enhancement by functionalized porous silicon devices. *Langmuir* 2008; **24**: 13765–13771.
11 Katz E, Willner I. Integrated nanoparticle-biomolecule hybrid systems: synthesis, properties, and applications. *Angew. Chem. Int. Ed.* 2004; **43**: 6042–6108.
12 Whaley SR, English DS, Hu EL, Barbara PF, Belcher AM. Selection of peptides with SC binding specificity for directed nanocrystal assembly. *Nature* 2000; **405**: 665–668.
13 Lee S-W, Mao C, Flynn CE, Belcher AM. Ordering quantum dots using genetically engineered viruses. *Science* 2002; **296**: 892–895.
14 Mao C, Flynn CE, Hayhurst A, Sweeney R, Qi J, Georgiou G, Iverson B, Belcher AM. Viral assembly of oriented quantum dot nanowires. *Proc. Natl. Acad. Sci. U.S.A.* 2003; **100**: 6946–6951.
15 Flynn CE, Mao C, Hayhurst A, Williams JL, Georgiou G, Iverson B, Belcher AM. Synthesis and organization of nanoscale II-VI semiconductor materials using evolved peptide specificity and viral capsid assembly. *J. Mater. Chem.* 2003; **13**: 2414–2421.
16 Reiss BD, Mao C, Solis DJ, Ryan KS, Thomson T, Belcher AM. Biological routes to metal alloy ferromagnetic nanostructures. *Nano Lett.* 2004; **4**: 1127–1132.
17 Estephan E, Larroque C, Bec N, Martineau P, Cuisinier FJG, Cloitre T, Gergely C. Selection and mass spectrometry characterization of peptides targeting semiconductor surfaces. *Biotechnol. Bioeng.* 2009; **104**: 1121–1131.
18 Estephan E, Saab MB, Larroque C, Martin M, Olsson F, Lourdudoss S, Gergely C. Peptides for functionalization of InP semiconductors. *J. Colloid Interface Sci.* 2009; **337**: 358–363.
19 Sanghvi AB, Miller KP-H, Belcher AM, Schmidt CE. Biomaterials functionalization using a novel peptide that selectively binds to a conducting polymer. *Nat. Mater.* 2005; **4**: 496–502.
20 Estephan E, Larroque C, Cuisinier FJG, Balint Z, Gergely C. Tailoring GaN semiconductor surfaces with biomolecules. *J. Phys. Chem. B* 2008; **112**: 8799–8805.
21 Estephan E, Bajoni D, Saab MB, Cloitre T, Aulombard R, Larroque C, Andreani LC, Liscidini M, Malvezzi AM, Gergely C. Sensing by means of nonlinear optics with functionalized GaAs/AlGaAs photonic crystals. *Langmuir* 2010; **26**: 10373–10379.
22 Naik RR, Brott L, Carlson SJ, Stone MO. Silica precipitating peptides isolated from a combinatorial phage display libraries. *J. Nanosci. Nanotechnol.* 2002; **2**: 95–100.
23 Naik RR, Stringer SJ, Agarwal G, Jones SE, Stone MO. Biomimetic synthesis and patterning of silver nanoparticles. *Nat. Mater.* 2002; **1**: 169–172.
24 Li CM, Botsaris GD, Kaplan DL. Selective in vitro effects of peptides on calcium carbonate crystallization. *Cryst. Growth Des.* 2002; **2**: 387–393.
25 Brown S, Sarikaya M, Johnson E. Genetic analysis of crystal growth. *J. Mol. Biol.* 2000; **299**: 725–732.
26 Ruffenach S, Moret M, Briot O, Gil B. Recent advances in the MOVPE growth of indium nitride. *Phys. Status Solidi A* 2010; **207**: 9–18.
27 Kyte J, Doolittle RF. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 1982; **157**: 105–132.
28 Lu Y-S, Ho C-L, Yeh A, Lin H-W, Hwo S. Anion detection using ultrathin InN ion selective field effect transistors. *Appl. Phys.Lett.* 2008; **92**: 212102-1–212102-3.

