An improved phage display methodology for inorganic nanoparticle fabrication

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Received (in Cambridge, UK) 20th February 2007, Accepted 30th April 2007
First published as an Advance Article on the web 11th May 2007
DOI: 10.1039/b702650a

The use of rolling circle amplification together with the addition of a wild-type control significantly improves the usefulness of phage display methodology as exemplified by the production of silver and platinum nanoparticles.

Phage display technology is a popular combinatorial technique for investigating the interaction of peptides with materials. The technique has found numerous applications, including the identification of peptides that bind to inorganic materials.[1,2]

A typical phage display technique uses a library of peptides expressed on the surface of filamentous bacteriophages. The single stranded DNA phages, M13, are composed of a protein coat with five copies of proteins pIII, pVI, pVII and pIX located at each end of the filament. In a typical phage display combinatorial library, some 2.7×10^9 random 12-mer peptides are expressed at the N-termini of pIII. This library is exposed to the target material in a process called panning and the binding phages are isolated by selectively eluting the unbound phages until only those that bind tightly to the material are finally eluted. The phages in this final elution may be amplified to produce a new library and the binding cycle is repeated up to three or even four times. The peptides expressed in the surface of the phages are identified by sequencing the phage DNA, as the DNA encoding the fusion resides within the virion.[1,2]

The peptides thus identified may then be synthesised and used in the control of chemical and physical processes such as the production of nanocrystals or particles of silver[3,4] and other metals. The mechanism of control of precipitation and particle size and morphology is not fully understood, but in the case of crystalline materials it has been suggested that there is selective binding of peptides at growing faces.[3]

Although the phage display method is very powerful and successful, conventional panning and analyses suffer from a number of disadvantages and shortcomings. In the process of amplification in generating the new libraries, there may be sequence biases toward particular sequences of peptides not necessarily reflecting the 'best' binding sequences. These biased sequences will become progressively more pronounced as the number of panning rounds increases. Also, by analysing only the eluted phages, those phages that bind so tightly that they are not eluted will be missed and the final binding peptides that are identified will not reflect the strongest peptide–substrate interactions. There is also a potential problem with the elution process in which surfactants and subsequently a glycine–HCl acid buffer are used. The use of acid to release the phage from the substrate may selectively liberate predominantly basic peptides which could again be enhanced with each round of panning. Additionally, the phage coat is proteinaceous and it cannot always be certain that bound phages are binding specifically through the expressed peptide rather than the protein coat. Finally, in applying the results of the phage display pannings, it is usually necessary to synthesise the selected peptides to screen their efficacy which is time consuming and expensive.

Stone and co-workers[4] made a significant advance in addressing some of these issues by demonstrating that the polymerase chain reaction (PCR) can be used directly with substrates, exemplified by metallic silver powder, to which phages remain bound after elution and the resulting DNA can be sequenced to enable the identification of the binding peptides. The use of this method enables tight binders to the silver to be identified efficiently and reduces the need for multiple rounds of panning.

The method does not resolve the potential issue of selective removal of basic clones by acid washing, but it does mean that the final peptides identified are definitely tight binders. Moreover, the PCR method gives fragments of phage DNA so performing a second round of panning requires several time-consuming manipulations. Further analysis and studies of the properties of the peptide also require expensive individual syntheses.

In this work, we introduce two novel variations on phage display technology. The first is the introduction of about 10% of wild-type phages into the panning mixture alongside the library (Ph.D.-12 in our case). This enables us to examine the bound phage DNA for non-specific as well as specific binding which can inform the future strategy. The second is the use of rolling circle amplification (RCA) using a polymerase of phi29 (in our case TempliPhi™) for the direct amplification of the DNA of phages bound to the substrate after multiple washings. RCA gives long strands of DNA that can be easily cut and ligated to produce multiple copies of circular phage DNA that may be transformed into *Escherichia coli* thereby readily generating phages in usable quantities for further experimentation. This saves considerable time and reduces the amount of manipulation of the DNA. To our knowledge, RCA has not been used in the identification of selected peptides in phage display experiments or with phages directly bound to substrates in the manner we describe below, although it has been used to amplify phage DNA[5,6,7] and Zhao *et al.*[8] have used RCA to produce DNA for direct interaction with gold and single-walled carbon nanotubes.[9]

Our method may be summarised as follows. The phage display library Ph.D.-12 (New England Biolabs), doped with wild-type phage, was exposed to a slurry of finely divided solid substrate

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suspended in Tris buffer saline (TBS) for one hour then the substrate was washed ten times with TBS containing 0.5% Tween-20 and the weak binders were eluted by several rounds of washing with glycine–HCl pH 2.2 buffer. A selection of the eluates and the solid remaining after the washings were analysed using PCR and subsequently the amplified products were loaded onto agarose gels. In this way the presence of wild-type (246 bp) and library phages (293 bp) were monitored (Fig. 1 shows the agarose gel for panning against silver and the band intensities give a good indication of the relative amounts of wild-type and library phages in each experiment). Assuming that some phages remain bound to the substrate after washing, the phage–target complex was directly subjected to RCA with TempliPhi™. The manufacturer's protocol recommends a denaturation step of 3 minutes at 95 °C to lyse the phages and release the plasmid DNA. Using this process, we required an input of a minimum of 10^6 phages, which is too high to be really useful. We managed to reduce the required number of input phages to a useful 10^4 by extending the denaturation step to 10 minutes at 95 °C in 5 μL of sample buffer. The reactions were then cooled to 4 °C and 5 μL of reaction buffer containing 0.2 μL of enzyme was added. The reactions were then incubated overnight at 30 °C. The amplified DNA was digested by incubating the mixture for 4 h at 37 °C with 2 μL of ACC65 I (10 000 units ml^{-1}) in Buffer 3 (New England Biolabs) containing 1 mg ml^{-1} bovine serum albumin (BSA) and then ligated using T4 DNA ligase (400 000 units ml^{-1}) in T4 Reaction Buffer (New England Biolabs). The ligated product was transformed in electrocompetent *E. coli* cells and plated into IPTG/Xgal plates. After overnight incubation at 37 °C, colonies were picked out and grown to obtain individual phages and DNA for sequencing.

We chose to exemplify the usefulness of the revised procedures by using phage display to aid the production of silver and platinum nanoparticles. Silver nanoparticle production using peptides and phages is well known[3,4] whereas there is no report of the use of peptides or phages being used for platinum nanoparticle fabrication. There is only one report of phage display technology in the identification of platinum binding peptides and that is a mention of unpublished work by the authors in a review.[10]

Ph.D.-12 library phages, together with 10% wild-type phages, were added to silver powder (Sigma-Aldrich) and our general procedure was followed.

After three acid washes, it was shown that very few wild-type phages remained bound to the silver, but library phages remained bound after five acid washes.

These tightly bound phages were amplified using RCA and, following the usual procedures, individual clones were obtained and sequenced. For silver, the binding peptide sequences showed similarities with those found by Stone *et al.*;[3] the motifs SS and PP appeared several times in the phages that resisted five acid washes. The silver binding peptides were enriched in glycine, leucine, proline and serine compared with the frequencies of these amino acids in the library. Of the 44 binding phages sequenced after five acid washes, we chose two for further experimentation. One expressed the peptide TVPPKAPRSSDL (Ag-22) and the other LTRPNHGNTVDT (Ag-28). Ag-22 contains both PP and SS sequences as found in some of Stone and co-workers' peptides[3] and Ag-28 was chosen randomly.

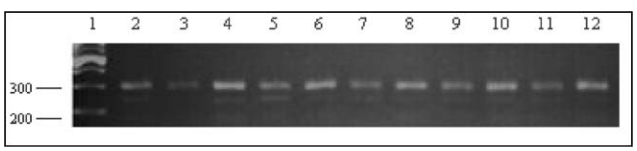

Fig. 1 Agarose gel of PCR products in the panning against silver: (**1**) 100 bp ladder; (**2**) non-bound phage; (**3**) first acid eluate; (**4**) solid washed with acid buffer once; (**5**) second acid eluate; (**6**) solid washed twice with acid; (**7**) third acid eluate; (**8**) solid washed three times with acid buffer; (**9**) fourth acid eluate; (**10**) solid washed four times with acid buffer; (**11**) fifth acid eluate; (**12**) solid washed five times with acid buffer.

The individual clones, wild-type phage and BSA as a non-specific protein source were incubated at room temperature with a silver nitrate solution (0.2 mM) in HEPES buffer pH 7.5 in the absence of reducing agents. A control experiment with no phages added was also performed. After 48 h, the solutions containing Ag-22 and Ag-28 phages (Fig. 2) were reddish coloured and homogeneous. The silver nitrate had been reduced to metallic silver nanoparticles as demonstrated by the characteristic plasmon resonance absorption of the silver nanoparticles as an intense peak at ~430 nm.[3] No precipitation or any evidence of reduction was detected in the negative control and BSA control. Wild-type phages were able to reduce the silver nitrate to metallic silver which precipitated from solution, but no characteristic peak at ~430 nm was observed in the supernatant solution, indicating that nanoparticles had not been produced. Transmission electron microscopy (TEM) analysis of synthesised nanoparticles was carried out. A droplet of each different solution of silver nanoparticles was placed on a copper grid with a carbon film and the solvent was allowed to evaporate. A sample of the silver slurry precipitated from the reaction with wild-type phages was also studied. Particles from the Ag-22 reaction exhibited triangular, quadrangular and spherical shapes. Those from the Ag-28 reaction were relatively uniformly spherical in shape with an average diameter of 20–50 nm. In the presence of wild-type phages, the aggregates were amorphous and bigger (Fig. 2A, B and C). This demonstrates that silver nanoparticles, with some control of morphology, may be prepared using selected Ph.D.-12 phages.

In similar experiments with platinum it was found that wild-type phages, alongside library phages, remained bound to the metal even after six acid washes (Fig. 3).

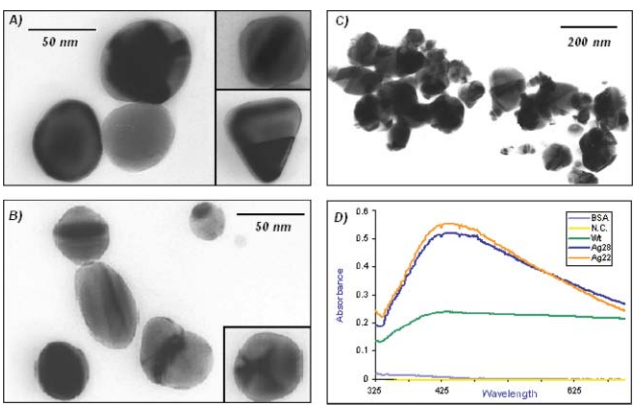

Fig. 2 Silver nanoparticles synthesised in presence of (**A**) Ag-22 clone; (**B**) Ag-28 clone and (**C**) wild-type phage. (**D**) UV-vis spectrum of silver nitrate solution after 48 h of incubation with Ag-22, Ag-28, wild-type (Wt), BSA and no phages (negative control (NC)).

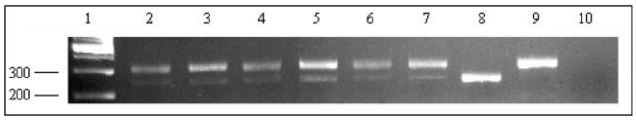

Fig. 3 Agarose gel of PCR products in the panning against platinum: (**1**) 100 bp DNA ladder; (**2**) fourth acid eluate; (**3**) solid washed with acid buffer four times; (**4**) fifth acid eluate; (**5**) solid washed five times with acid buffer; (**6**) sixth acid eluate; (**7**) solid washed six times with acid buffer; (**8**) wild-type positive control; (**9**) Ph.D. library positive control; (**10**) negative control.

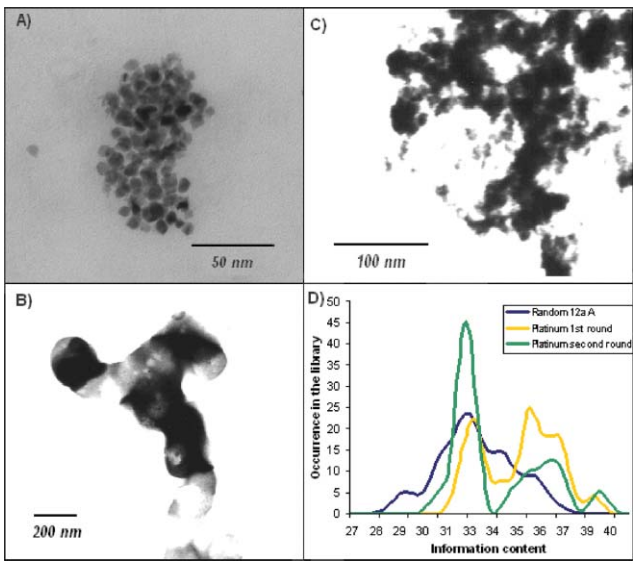

Fig. 4 Platinum nanoparticles synthesised in the presence of (**A**) Pt-41 clone; (**B**) wild-type phage and (**C**) in absence of phage. (**D**) Information content distribution of platinum peptides compared to the original library.

In this case, in an attempt to improve selectivity, a second round of panning was carried out. The peptide cohort from the second round was noticeably lower in overall 'information content' (a measure of the probability of a peptide sequence being randomly chosen or specifically selected) (Fig. 4D) as revealed by the INFO program.[11] However, the information content of the second round has a pronounced bimodal nature with the major enhancement relative to the first round being of low information content peptides that presumably result from sequence bias. This limits the usefulness of the second round of panning in this case and we decided to perform the following experiments with clones only from the first round of panning. The peptide cohorts for selective platinum binding as determined from the first round were somewhat enriched in glutamic acid, proline, phenyl alanine and serine relative to the library peptides.

Random library phages from the first round of panning were chosen to demonstrate the ability or otherwise of phages to prepare platinum nanoparticles. The phages were added to a solution of chloroplatinic acid (1 mM) in phosphate buffer at pH 7.0 and the mixtures were incubated overnight at 4 °C. 10 μL of sodium borohydride (25 mM) was added slowly to reduce the metal cation. The solutions were incubated overnight at 4 °C.

During this period, metallic platinum precipitated in the control reaction as expected under these reducing conditions (wild-type phage and negative control). Solutions containing phages that expressed peptides SRLTHSNYATPT (Pt-41), EHTNPILSHTHN (Pt-14) and QSFSTNVLHTHH (Pt-1.2) behaved differently and did not produce a precipitate even after 5 days, but the dark grey/black solutions did exhibit strong, broad, continuous plasmon resonances, showing that platinum nanoparticle formation had occurred.[12] We compared our sequences with those found by Sarikaya[10] using the sequence alignment program clustalW[13] which showed good similarity between the two series.† Two of the peptides (Pt-14 and Pt-1.2) that were able to control platinum nanoparticle formation contain the motifs HTN and HTH which were also found in the 7-mer platinum binding peptide sequences reported by Sarikaya.[10] The third phage, Pt-41, was chosen randomly from the pool of peptides that remained bound to the solid after six acid washes.

The platinum nanoparticles produced by the phages were characterised using electron microscopy by evaporation of the solution as before. The TEM images (Fig. 4A) showed that the particles were not completely separated from each other, but they are not coalesced into tight agglomerates. The platinum nanoparticles obtained are very small at around 3–4 nm and they are roughly spherical. Without the presence of platinum-binding phages, the precipitated platinum comprises large unresolved particles.

Again, metal-binding phages that are produced readily and rapidly by using RCA have been shown to aid metal nanoparticle production and to exercise some control of size and morphology.

Thus, we have demonstrated that rolling circle amplification combined with the use of wild-type phages, for monitoring the specificity of binding to solids, increases the ease, effectiveness and speed of phage display technology.

Notes and references

† Full sequence data, comparisons, and results for gold, platinum and silver nanoparticles will be published in the full paper which is in preparation.

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