

This item was submitted to Loughborough's Institutional Repository (<https://dspace.lboro.ac.uk/>) by the author and is made available under the following Creative Commons Licence conditions.



For the full text of this licence, please go to:
<http://creativecommons.org/licenses/by-nc-nd/2.5/>

DOI: 10.1002/sml.((please add manuscript number))

M13 Bacteriophage-Activated Superparamagnetic Beads for Affinity Separation

Julien Muzard¹, Mark Platt¹ & Gil U Lee^{1,*}

¹ UCD Centre for Nanomedicine, School of Chemistry & Chemical Biology - University College Dublin Belfield, Dublin 4, Ireland

* Correspondence should be addressed to gil.lee@ucd.ie.

Supporting Information is available on the web at <http://www.small-journal.com> or from the authors.

Abstract

The growth of the biopharmaceutical industry has created a demand for new technologies for the purification of genetically engineered proteins. The efficiency of large-scale, high-gradient magnetic fishing could be improved if magnetic particles offering higher binding capacity and magnetization were available. This article describes several strategies for synthesizing SPMs that are composed of a M13 bacteriophage layer assembled on a superparamagnetic core. Chemically cross-linking the pVIII proteins to a carboxyl functionalized SPM produced highly responsive superparamagnetic particles with a side-on oriented, adherent virus monolayer. Also, the genetic manipulation of the pIII proteins with a His₆ peptide sequence allowed reversible assembly of the bacteriophage on a nitrilotriacetic acid functionalized core in an end-on configuration. These phage-magnetic particles were successfully used to separate antibodies from high-protein concentration solutions in a single step with a > 90 % purity. The dense magnetic core of these particles makes them five times more responsive to magnetic fields than commercial materials composed of polymer-iron oxide composites and a monolayer of phage could produced a 1000 fold higher antibody binding capacity. These new bionanomaterials appear to be well-suited to large-scale high-gradient magnetic fishing separation and promise to be cost effective as a result of the self-assembling and self-replicating properties of genetically engineered M13 bacteriophage.

Keywords

Bionanomaterial, M13 filamentous phage, affinity separation, downstream processing, superparamagnetic bead, high gradient magnetic separation

Formatted: Not Highlight

Formatted: Not Highlight

1. Introduction

A shift is taking place in the pharmaceutical industry whereby an increasing portion of the most successful new therapeutics is being derived from genetically engineered antibodies ^[1].

The efficient, large-scale production of high-purity recombinant proteins has thus become an important technical goal of the biopharmaceutical industry. Downstream processing (DSP) remains a bottleneck in the production of recombinant human biotherapeutic proteins, accounting for up to 80% of the manufacturing costs in some cases. Much of this cost can be attributed to the expense of chromatographic matrices, with bioaffinity types featuring protein ligands being at least an order of magnitude more expensive than ion exchange matrices employing the same base matrix. In specific, protein A is the standard for antibody purification due to its high-affinity for a wide-range of antibody sub-types and genetically engineered ability to withstand strong chemical conditions used to sterilize the affinity separation matrix ^[3].

Superparamagnetic microparticles (SPMs) have been widely used for single-molecule detection ^[4], cell sorting ^[5], nucleic acid together with protein purification ^[6], and *in vivo* magnetic resonance imaging ^[7]. The manipulation and separation of biomolecules and cells using SPMs offers a significantly faster rate of separation than traditional chromatography.

High-gradient magnetic fishing (HGMF) is an integrated bioprocess product capture technology, employing specialised magnetic adsorbent particles in combination with high-gradient magnetic separation equipment, to fish out and partially purify target bioproducts from complex unclarified bioprocess liquors ^[6]. However, efficient large-scale HGMF requires that the SPMs have both a high magnetic moment and a high analyte binding capacity. These properties cannot be obtained with a simple spherical bead as the magnetic moment scales as $[r^3]$ while surface area/volume *ratio* scales as $[r^{-1}]$ ^[8]. This has led to the development of more complicated SPM structures where increased binding capacity is created through roughened

Formatted: Not Highlight

Formatted: Not Highlight

Formatted: Not Highlight

surfaces^[9, 10].

Natural scaffolds have recently gained attention as a promising approach for building nanostructures due to their well-defined molecular shape, propensity to self-assemble, and ease of modification through genetic engineering^[11-13]. In particular, the bacteriophage has been extensively used for molecular engineering^[14, 15] and other applications^[16, 17]. Phage-based materials have been developed for biomedical applications including biosensing^[18, 19], affinity purification^[20-22], *in vivo* imaging^[23-25], drug/gene delivery, and antibiotic therapies^[26, 28]. The M13 bacteriophage is 6 nm in diameter and ~900 nm long virus^[29] with a large surface area of 18,700 nm²^[30]. From a structural perspective, the virion is dominated by the 2,700 copies of the pVIII coat protein, representing 87 % of its total mass and 99 % of the total virus surface (Figure 1a). The N-termini of these proteins are flexible, hydrophilic domains, located on the outer surface of the phage and are able to support peptide inserts of up to 8 amino acids for display on every pVIII unit^[31] (Figure 1b). The first eight amino acids of the pVIII subunit of wild-type M13 virus include two amino groups and three carboxyl groups that are exposed to solvent on the surface of the virion, thus they can be chemically modified without compromising the virion integrity and its infectivity^[32] (Figure 1b and c). Five copies of each of the four minor coat proteins (pIII, pVI, pVII and pIX) close off the ends of the bacteriophage structure^[33]. The N-terminal part of the pIII protein is the most widely used system to display foreign (poly)peptides, and random libraries on the phage surface (phage display technology, for review^[34, 35]). The abundance of chemically addressable functionalities on the phage surface and the ability to manipulate the virus genome *via* molecular biology techniques have been used to create recombinant polymeric scaffolds^[30, 36]. The phage structure is extraordinarily robust, being resistant to heat, organic solvents, acidic conditions, alkali conditions and even 6 M urea^[29, 37]. Purified phage particle can also be stored at moderate temperature without jeopardizing its infectivity and/or binding

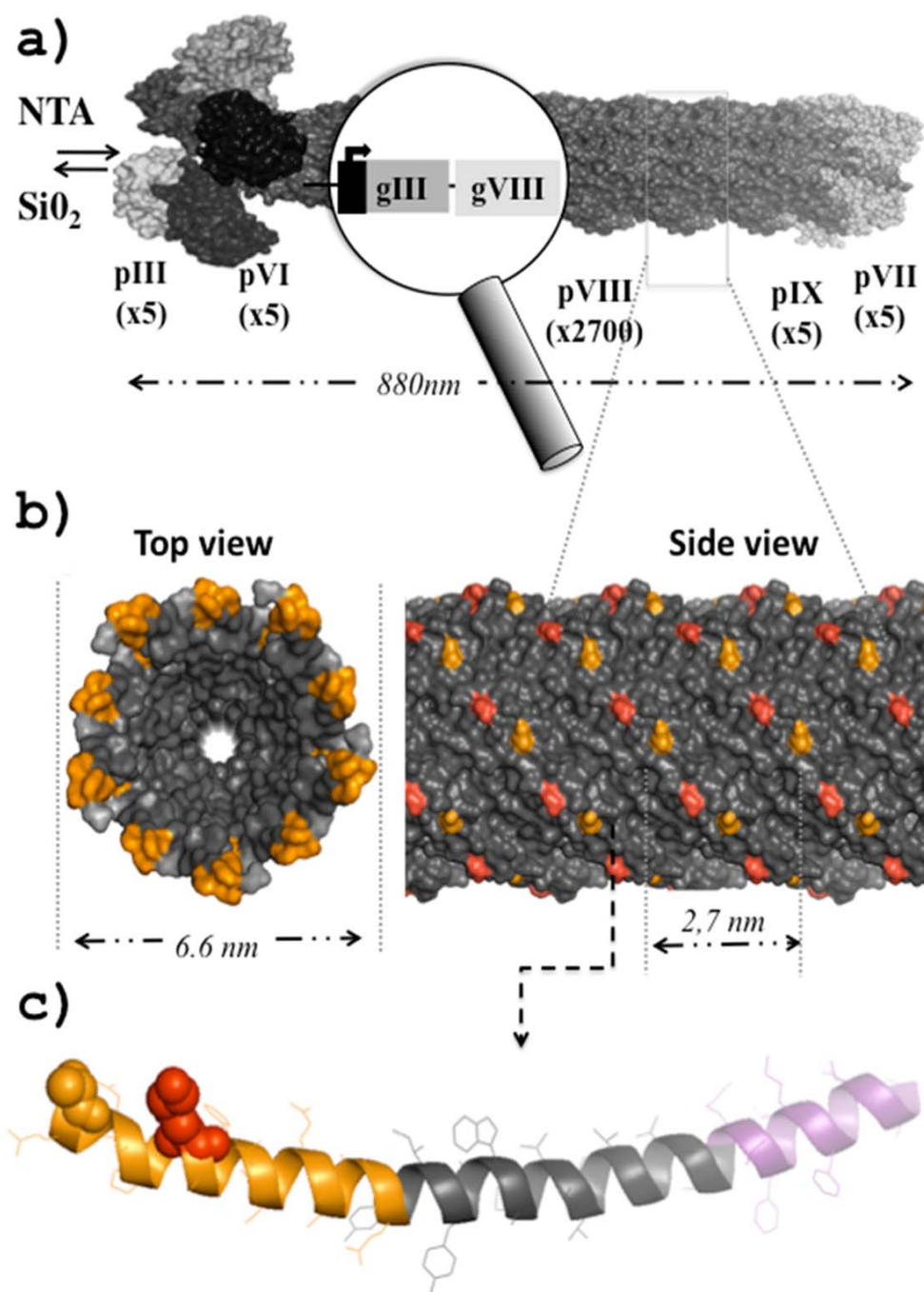
activity [21, 22, 31, 38].

This article describes strategies for modifying the M13 phage to allow it to be used as a multi-functional material for HGMF affinity separation. Figure 1 shows a schematic of the M13 phage coat proteins and genetic structure that have been used to modify the phage with functional groups for simultaneous assembly and affinity separation on SPMs. The pIII and pVIII phage coat proteins have been genetically and chemically modified, respectively, to allow the phage to assemble at high densities on SPMs. These high surface area bionanomaterials have proven to be highly functional for magnetically activated antibody and protein affinity separation. To the best of our knowledge, this is the first report detailing the successful grafting of M13 onto SPMs for use as a potential affinity matrix in HGMS.

2. Results

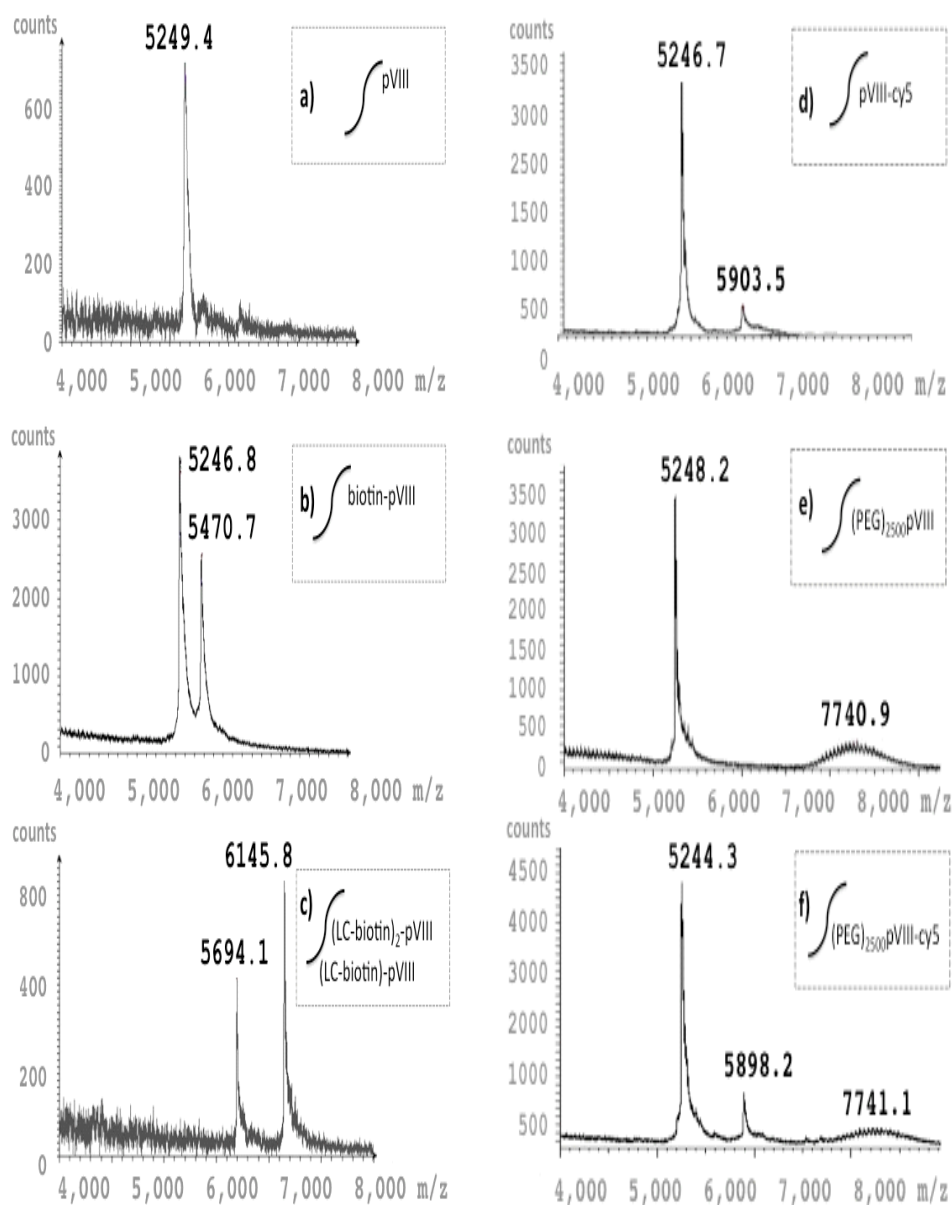
Genetic and chemical modifications of M13 phage coat proteins

Figure 1a presents the strategies that were used to genetically modify the pIII coat proteins with either a –HHHHHH (M13His) or –CHKKPSKSC^[39, 40] (M13Si) peptide to allow the phage to be assembled on SPMs that have been coated with either nitrilotriacetic acid (NTA) or silica, respectively. These peptides were inserted at the N-terminus of the five pIII proteins by synthesizing the appropriate double-stranded DNA sequence and cloning it between the *EagI* and *KpnI* restriction sites of the pM13KE plasmid (sequences details are presented in the *Experimental Section*). The integrity of the final constructs was confirmed by sequencing the phage DNA using the -96gIII primer and propagating the bacteriophage in *E. coli* ER2738. The M13 mutants were grown and purified at a high yield of 20-30 mg per litre of *E. coli* culture, which is similar to the level of expression that was achieved for the wild-type M13 virus. This result confirmed that the pentavalent display of the foreign binding peptides did not negatively influence the ability of the M13 viruses to infect *E.coli* cells.



The pVIII coat protein (Figures 1b and 1c) was chemically modified to allow side-on linkage of the phage to the SPMS. Figure 2a shows a MALDI-TOF mass spectra of the M13 phage

with a single peak at approximately 5,250 m/z, which was consistent with the mass of the amino-acid sequence of the protonated pVIII coat protein of wild-type M13 ^[41]. Figure 2b shows the spectra of a phage that has been reacted with N-hydroxysuccinimido-biotin (NHS-biotin). At low levels of modification of the M13 phage with NHS-biotin, which we define as ~400 biotins per phage (as determined by the HABA assay which is described in the *Supplementary Section*), two peaks were observed at 5,246 and 5,470 m/z. The first species was wt-pVIII and the second peak was identified as pVIII coat protein modified with a single biotin group. Complete reaction of the phage with NHS-LC-biotin resulted in a high-level of functionalization of the pVIII protein with ~2,500 biotin molecules per phage. Mass spectrometry measurements of phage saturated with a high-level of biotin (Figure 2c) indicated that the pVIII proteins were fully modified with either one or two biotin groups.



The pVIII protein on M13 was further chemically modified with several other molecules. Figure 2d shows the mass spectra of M13 that was reacted with NHS-Cy5 in which can be seen the pVIII protein and a single 650 Da Cy5 molecule linked to the pVIII protein. Fluorescence measurements of the Cy5 labelled phages indicated that as many as ~600 Cy5

molecules reacted with a single phage (please see *Supplementary Section*). A polyethylene glycol polymer of 2,500 Da nominal molecular weight (PEG₂₅₀₀) was also covalently coupled to the pVIII protein using the amine reactive NHS chemistry. As shown in Figure 2e, a series of peaks were observed in the mass spectra of the pVIII-PEG complex that are characteristic of the polydispersity of the PEG polymer. Dual labeling of the M13 has also been demonstrated with Cy5 and PEG₂₅₀₀. The first peak (Figure 1f) at approximately 5,900 m/z corresponds to the attachment of the Cy5 with a coverage of ~ 200 molecules per phage and a second set of peaks at approximately 7,740 Da were consistent with the PEG₂₅₀₀ moieties.

Synthesis and characterization of M13-superparamagnetic particles

Superparamagnetic microparticles were synthesized using emulsion templated Fe₃O₄ nanoparticle assembly ^[8], resulting in particles with an average size of 1 micron, a CV of 20 %, and a saturation magnetisation of approximately 55 emu/g (*Supplementary Section*, Figure S2). The M13 phages were non-covalently or covalently linked to these SPMs attaching by using either the engineered pIII or pVIII coat proteins. Non-covalent capture with the pIII protein was achieved using M13Si or M13His to bind to the silica or NTA coated particles, respectively. Controlled phage capture was performed using monoclonal anti-pIII antibody coated SPMs. Immobilization through the pVIII protein was achieved using either direct covalent coupling to carboxyl-functionalized particles or specific attachment, i.e., biotin-phage with neutravidin coated SPMs.

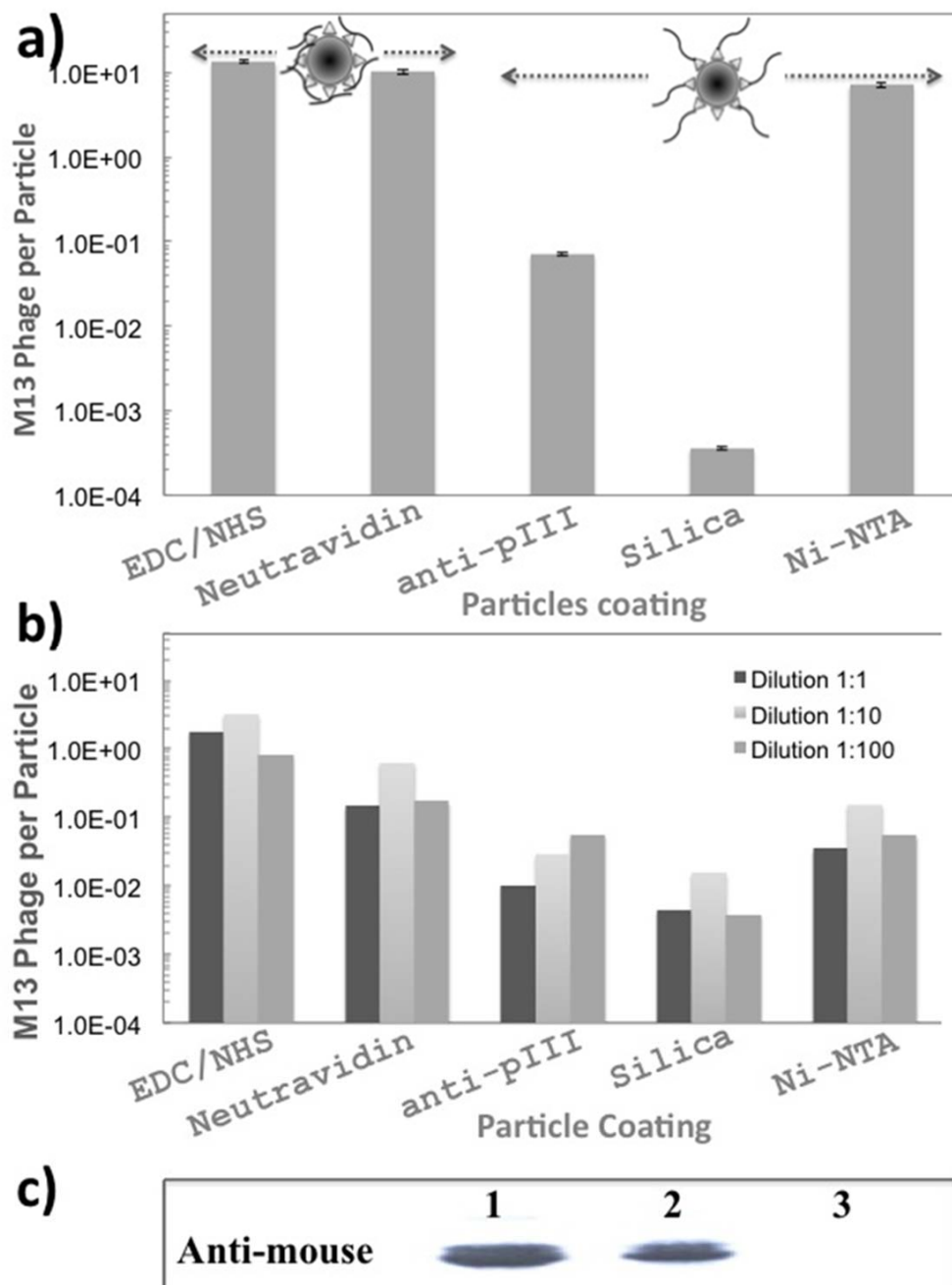
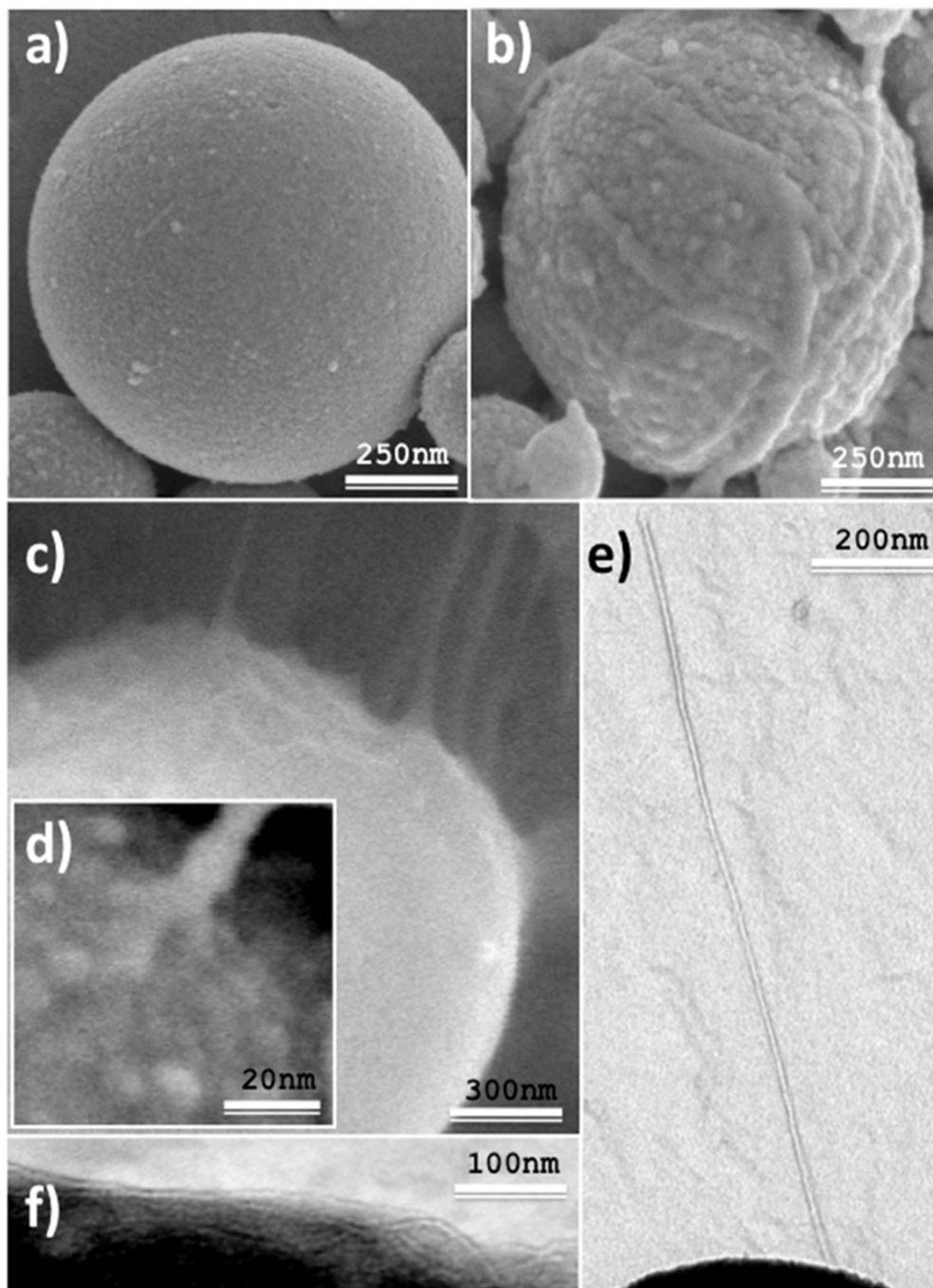


Figure 3 presents the fluorescence and colorimetric measurements of the phage bound to the SPMs using the five strategies at a fixed reaction concentration of 10^{10} phage to 5×10^8 SPMs in a single mL reaction volume. Both fluorescence and colorimetric measurements

demonstrated similar trends, i.e., EDC/NHS \approx biotin-neutravidin > Ni-NTA > anti-pVIII > silica, although the absolute number of phage per SPM measured by fluorescence was consistently higher than the colorimetric results (this systematic difference was attributed to the calibration of the colorimetric measurement assay, which underestimated the amount of phage on the particles). Direct covalent coupling of the phage to the NHS functionalized SPMs using the pVIII surface coat proteins produced the highest coverage of phage. The M13-biotin-neutravidin particle assembly strategy also resulted in high immobilization efficiency, but the anti-pVIII antibody immobilization approach resulted in a significantly lower coverage of M13. For non-covalent immobilization, M13His were detected at coverages approximately half of those using the NHS chemistry. The lowest particle coverage was obtained using the genetically modified M13Si phage.



The physical conformation of the M13 structure on the SPMs was monitored using electron microscopy. Figure 4a shows a SEM micrograph of a single particle with a relatively smooth

surface. Figure 4b shows M13 covalently immobilized onto a bead through the EDC-NHS coupling chemistry in which multiple filamentous structures can be clearly observed tightly attached to the surface of the beads. Figures 4c and 4d indicate that the dominant mode of assembly of M13His phage to the NTA coated magnetic particles was through the end of the phage. This is consistent with a specific interaction in which the five copies of the pIII protein bind to functional NTA groups on the SPM surface.

The phage-particle interaction was studied at higher resolution with TEM. Figure 4e shows a micrograph of a single M13His phage projecting normal to the surface of the black SPM at the bottom of the image. The orientation of the phage is consistent with a specific attachment of the genetically modified pIII protein to this single SPM. Figure 4f shows that the attachment of viruses to neutravidin functionalized SPMs through biotinylated functionalized pVIII protein resulted in filamentous structures tightly packed to the neutravidin SPM surface. In order to test the utility of M13His phage-SPMs as a potential HGMS affinity matrix, we carried out affinity separation of the anti-pVIII protein monoclonal antibody as a model analyte. These SPMs have a very high magnetic moment, thus separation was very rapid, even at low densities of SPMs, typically < 6 mins. The separation was performed in a concentrated solution of bovine serum albumin (BSA, 20 mg/mL) and the bound protein was eluted from the magnetic matrix using an acidic buffer. Gel electrophoresis followed by MALDI-TOF analysis was performed on the purified fraction to estimate the relative purity of the sample. BSA was not detected in product effluent from M13His-particles thus we estimated the purity to be > 90 %. The presence of antibody in the eluted fraction was confirmed by immunoblotting using a specific anti-IgG probe (Figure 3c). We measured the binding capacity of the M13His-SPMs to be 10-15 µg of antibody removed for one mg of SPM, i.e., 7.7×10^7 particles. This is a similar to the binding capacity resulting from protein monolayers on the SPMs (*e.g. Dynal, Invitrogen*). However, in this specific measurement only ten M13

Formatted: Not Highlight

phage were bound to each SPM and the equilibrium coverage of the phage can theoretically reach 10,000 phage per particle. These results indicate that the binding capacity of the M13His-SPMs could reach 1000 times higher than that of direct binding chemistries. The phage-magnetic particles remained functional in their ability to bind the anti-pVIII antibody for up to 5 brief acidification steps given they were stored at low temperature.

Formatted: Not Highlight

3. Discussion

In this article we describe the assembly of M13 phage on SPMs using genetic engineering and covalent chemical modification to determine the potential of these highly sophisticated bionanomaterials for large-scale affinity separation. The side-on assembly of the M13 phage on SPMs was achieved by either direct covalent reaction with the SPMs or the specific molecular interaction of the pVIII coat protein with a protein on the surface of the SPMs. Fluorescence and colorimetric measurements of the binding of the phage to the SPMs, shown in Figure 3, demonstrated that both assembly strategies were successful. However, the phage coverage was found to be highest for assembly strategies that used strong phage-SPM interactions, i.e., EDC-NHS \approx neutravidin-biotin > anti-pVIII bond. The maximum number of phage per SPM, i.e., approximately 13 M13 phages per SPM of the EDC-NHS chemistry, resulted in the coupling of 65 % of the phage in the initial reaction to the SPMs. Electron microscopy images of the SPMs treated with phage, Figures 4b and 4f, revealed that the phage were tightly bound to the bead surface, consistent with the attachment of the phage to the beads at multiple points. The SEM images also revealed that EDC-NHS and biotin-neutravidin chemistries produced a network of phage on the SPMs that, while not fully dense, was sufficiently packed such that the majority of the phage crossed over at least one other phage at some point. These images suggest that strong phage-SPM interactions are required to allow phage to bend around and cross over adjacent phage while still remaining tethered to the bead.

The end-on assembly of the filamentous M13 on the SPMs was achieved by genetically modifying the five pIII proteins with specific peptide sequences that recognize chemistries on the surface of SPMs. Measurements of the amount of phage bound to the SPMs, shown in Figure 3, demonstrated that both peptides can be used to immobilize the phage on the surface of the particles and that the NTA-His₆ bond produced a surface coverage significantly higher than M13Si. Electron micrographs, Figures 4c, 4d and 4e, confirm that these phage were immobilized in an end-on configuration, providing strong evidence that the assembly is driven by the specific molecular interactions of the pIII proteins with the microparticle surface. Surprisingly, though, very little M13Si phage was detected on the silica coated particles after incubation with PBS-BSA, which suggests that the M13Si-microparticle assembly would not be stable in the presence of protein. In contrast, the M13His phage-microparticle binding was found to be stable in PBS-BSA. The binding forces of the His₆-NTA and peptide-Si bonds to their respective targets have been reported to be of the same order of magnitude, i.e., the peptide-Si bond has an affinity in the range of 8.7 μM ^[40] and the His₆-NTA bond is 10 μM ^[42]. The introduction of multiple bonds into the assembly produced a geometric increase in the overall affinity (avidity) and two His₆-NTA bonds have demonstrated a half-life of hours in serum ^[42]. This suggests that the stability of the phage-SPM structure resulted from factors other than bond energy. Non-specific adsorption of proteins is known to readily take place on silica surfaces and lead to the preferential coverage of the strongest binder ^[43]. This suggests the M13Si phage may be displaced from the silica surface due to the competitive adsorption of BSA with the glass. Non-specific protein adsorption was expected to be less of an issue for the His₆-NTA chemistry as the NTA groups were grafted through a PEG monolayer that is known to inhibit non-specific adsorption and this was shown to be the case.

Formatted: Not Highlight

Formatted: Not Highlight

A highly useful feature of the M13 phage for affinity separation is that the filamentous body is made of 2,700 pVIII proteins that can be modified to create a high density of functional groups ^[43]. In this study, the reactive residues (e.g. lysine-8) on the pVIII protein were targeted for modification with NHS groups, which allowed a highly specific nucleophilic reaction to take place in aqueous solution at room temperature. This chemistry was used to functionalize the pVIII coat proteins with biotin, Cy5 and much larger macromolecules, i.e., 2,500 Da molecular weight PEG. Mass spectrometry demonstrated that NHS-biotin can react with one or more amine reactive groups accessible to the surface of the phage body, as shown in Figures 2b and 2c. Colorimetric measurements indicated that high reaction efficiencies could be achieved, i.e., 99 % of the pVIII proteins could be functionalized with biotin groups. A significantly lower fraction of the pVIII coat proteins was modified by the larger Cy5 molecule, i.e., fluorimetry detected that approximately 25 % of the pVIII proteins were bound with Cy5, with even lower coverage for the PEG.

The phage-SPMs seemed to be promising materials for HGMS affinity separation. High-salt and acidic buffers as low as pH 2.2 are commonly used to elute target proteins from affinity separation supports ^[39]. The phage-SPMs withstood these same relatively harsh conditions without being compromised, which is consistent with the observation that wild-type phage retain their infectivity after a brief exposure to these conditions. In this study, anti-pVIII antibodies were successfully recovered at high yield on a single step separation. Also, phage-SPMs could be used up to five times after being submitted to the (low pH) elution method and NaOH sterilization. We finally demonstrated that the M13 virion could also be modified with PEG, a hydrophilic polymer known to inhibit aggregation of colloidal particles and protect proteins from enzymatic degradation ^[44]. These results demonstrate that M13-linked magnetic particles can be used to separate specific proteins in a single step and simultaneously tolerate the harsh conditions typically inherent in affinity separation.

Formatted: Not Highlight

Formatted: Not Highlight

4. Conclusions

These experiments have demonstrated that phage-SPMs complexes can be created if the M13 phage is chemically or genetically modified to drive assembly on SPMs in a side or end-on configuration. Several observations have been made concerning the successful grafting of phage onto SPMs. First, strong specific molecular or covalent interactions must be used to drive functional assembly. The use of lower-energy assembly strategies resulted in lower phage coverage. Second, the surface chemistry of the beads must be controlled to minimize non-specific interactions that block assembly of the phage on the SPM and decrease the purity of the final product. While the simplest strategy is chemical modification, either *via* EDC/NHS or biotin produced, this method resulted in some loss of functionality. The most convenient method for assembly of phage to the beads is end-on attachment using genetically modified pIII proteins using the Ni-NTA binding peptides. Though large-scale HGMP has not been demonstrated in this work, the described adsorbent meets many of the criteria identified by Franzreb et al. as necessary for use in HGMP applications ^[10]. In specific, the M13His-SPM construct promises to be highly useful for large-scale affinity HGMS of high-value biomaterials and biopharmaceuticals as 1) it is likely to be highly response to magnetic fields, 2) affinity separation can be executed in a single step, 3) the pVIII protein can be easily genetically (or chemically) modified, and 4) the pVIII protein is present at very high copy numbers. This material also promises to be more cost effective than the conventional polymeric or glass affinity separation materials due to the advantageous self-replicating and self-assembling properties of the bacteriophage virus.

Formatted: Not Highlight

5. Experimental Section

Design and construction of mutant M13 phage

Standard phage display molecular biology procedures ^[39] were used to pentavalently express

six histidine residues (M13His) or CHKKPSKSC (Si-M13) peptides fused to the N-terminus of the pIII minor coat protein of the wild-type (wtM13) virus ^[41]. The sequence details and restriction sites used for cloning are shown in Figure 1 of the *Supplementary Section*. Double-stranded DNA sequences were inserted by Genscript (Piscataway NJ, USA) in the pM13KE plasmid (New England Biolabs, Hitchin, Hertfordshire, UK) between the EagI and KpnI sites and treatment with T4 ligase yielded pM13KE-His (M13His) and pM13KE-Si (M13Si). The ligation products were subcloned into TG1 cells and plated on LB plates in the presence of 20 µg/mL isopropyl-β-D-1-thio galactopyranoside (IPTG) and 20 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to generate blue phage plaques. The integrity of the final vector constructions was confirmed by DNA sequencing (Genscript, Piscataway, USA) using the ~96gIII primer (New England Biolabs).

Large-scale amplification of M13 viruses

The M13 virus was grown and purified using standard microbiology and biochemical protocols. An *E. coli* ER2738 (NEB, Hitchin, Hertfordshire, UK) culture was grown in 1 L of Luria Bertani (LB) media (Fisher Scientific, Dublin, Ireland) supplemented with tetracycline to mid-log phase and infected with approximately 10¹² pfu of M13 bacteriophage. This culture was incubated at 37 °C and then shaken at 180 rpm for 5 h. The virus was recovered by: centrifuging the culture to remove bacterial cells, precipitating with PEG/NaCl (20 % PEG and 2.5 mol/L NaCl), and reconstituting in phosphate buffer saline (Dubecco-PBS; 0.137 M Sodium Chloride, 0.0027 M Potassium Chloride, and 0.0119 M Phosphates), pH 7.4. The typical yield was ~20 mg of purified M13 from one litre of infected bacteria. The phage solution was further purified by filtration through 0.45 µm pore-size membrane. The concentration was evaluated spectrophotometrically as described in the *Supplementary Section*.

M13 infectivity was determined by plating and plaque counting. Defined dilutions of M13

phages were plated on LB agar plates with an ER2738 cell lawn supplemented with 20 µg/mL IPTG, 20 µg/mL X-Gal, and 10 µg/mL tetracycline. Plates were examined for the presence of blue plaques after 18 h of incubation at 37°C.

Surface modification of M13

In a typical chemical modification reaction, a stock solution of M13 phages was diluted to $\sim 5 \cdot 10^9$ phage/mL with PBS buffer. Biotinylated phage (M13-biot) was prepared by reacting the M13 phage with 5 mg of either NHS-biotin (N-hydroxysuccinimidobiotin) or NHS-LC-biotin (succinimidyl-6-(biotinamido)-6-hexanamido hexanoate (Pierce, Thermo Scientific, Dublin, Ireland) at 4 °C. Reactions were stopped by adding Tris to a final concentration of 50 mM. Modified phage particles were dialysed (Centricon, Millipore, MWCO 10,000 Da) against PBS to remove unreacted reagents. A PEG/NaCl precipitation was subsequently performed to purify M13 virus followed by re-suspension in PBS. Biotin conjugation to the phage surface was evaluated using the HABA (Sigma-Aldrich Ireland Ltd), which has been described in the *Supplementary Section*. For high surface coverage, viruses were diluted to $\sim 1 \cdot 10^7$ /mL before modification. For dual modification, M13 was first labelled with NHS-Cy5 (Amersham, Buckinghamshire, UK) to generate ~ 400 dyes/phage particle (*Supporting Information*). The number of Cy5 per phage was determined spectrophotometrically at 650 nm using molar extinction coefficient for Cy5 of 250,000. After dialysis against PBS, the Cy5-M13 ($\sim 5 \cdot 10^8$ /mL) was further modified using PEG-NHS (7 mg in carbonate buffer, pH 8.2) (Rapp Polymere GmbH, Tübingen, Germany) for 16 h to generate bifunctional M13 particles.

MALDI-TOF MS of M13 subunit

M13 was directly spotted onto a MALDI chip at a concentration of 1 mg/mL in MS grade alpha cyano-4-hydroxycinnamic acid in 70 % acetonitrile, 30 % water and 0.1 % TFA as the matrix. The phage was desalted using a µ-C18® (Millipore, Tullagreen, Ireland) ZipTip. The

samples were analyzed using a MALDI-TOF mass spectrometer (LaserToF TT, Scientific Analytica Instruments, UK) operating at 20 kV in linear mode.

SPM synthesis and M13 immobilization on the SPMs

One micron sized SPM with a 20 % CV was synthesized according to a procedure developed by Shang et al ^[9]. The particles were functionalized with NHS, NTA, silica, neutravidin or anti-pIII antibody, using synthetic procedures described in the *Supplementary* Section. The physical properties of the synthesized particles were characterized with a superconducting quantum interference device (MPMS, Quantum Design, Magnetic properties measurement system), light scatter measurements, and electron microscopy (*Supporting Information*).

Immobilization of genetically modified bacteriophages (M13His, M13Si) on the SPMs was performed by overnight incubation of 1 mL of 10^{10} pfu/mL modified M13 viruses with 500 μ L of $\sim 1.10^9$ beads/mL magnetic beads, with gentle shaking at room temperature on an orbital rotator. Wild type, unmodified M13 bacteriophages (wtM13) were used as a control. For immobilization of chemically modified phages onto particles, incubation was decreased to 3 hours in the same conditions. The unbound phages were removed by washing the magnetic particles six times with phosphate buffered saline Tween 20 solution (PBST). M13 linked to magnetic particles were collected using a permanent magnet, re-suspended in 1 mL of PBST buffer, and stored at 4 °C until they were used. Phages were enumerated in the initial solution, supernatant/washing solutions, and the number of phage immobilized on magnetic particles was estimated by subtraction.

Phage coverage measured by solid-phase immunoassays and flow cytometry

Solid-phase colorimetric immunoassays were conducted on the magnetic particles using anti-M13-HRP (GE Healthcare, Buckinghamshire, UK). The colorimetric response of the assay was calibrated against defined concentrations of M13 immobilized on surfaces. The wells of a 300 μ L microtitration plate were coated with increasing concentrations of wt-M13 phages by

incubation in PBS buffer overnight at 4°C. Non-immobilized phages were discarded and free sites were blocked with 2.5 % (wt) skimmed milk powder in PBS for at least 2 h at room temperature. Colorimetric assays were performed by washing the plates with 0.2% Tween 20 in PBS, incubating with 100 µL anti-M13-HRP (1/5000) for 2 h, and reacting with the 3,3',5,5'-tetramethylbenzidine solution (One-Step-TMB, Pierce, ThermoFisher, Dublin, Ireland). The relative HRP concentrations were determined by stopping the reactions with 30 µL of a 4M sulphuric acid solution and measuring the absorbance at 450 nm (BIO-Tek ELISA reader). M13 linked-superparamagnetic particles were detected using the same method and then compared to the titration curve obtained from the surfaces.

Fluorescence assays were conducted on the SPMs using FITC-anti-M13 (Abcam, Cambridge, UK). The SPMs were incubated at a concentration of 1.10^8 /mL with know amount of phage in solution follow by the addition of 10 µL of FITC-anti-M13 at room temperature for 2 hours. After washing three times in PBST, the particles were analyzed on a FACScalibur flow cytometer (BD Biosciences, Oxford, UK). Up to 5×10^4 particles were counted and analyzed for fluorecence intensity using dual color lasers at 525 nm and 660-675 nm.

Separation and Immunoblotting

Monoclonal anti-pVIII antibody was diluted in a concentrated BSA solution (2.5 %) and the mixture was incubated for 16h at 20°C with 1 mL functionalized magnetic particles (10 mg.mL). After extensive washing with PBST until A280 reached 0.001, bound proteins were eluted in fractions of 100 µL of pH 2.2 (glycine, 100 mM) and fractions > 0.1 being submitted to a MALDI-TOF assay, SDS-PAGE analysis and Western-Blotting. After reaction with anti-pVIII epitope antibody, the elution fraction (Glycin 50 mM, pH 2.2) were electrophoresed by a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (semi-dry, 20 V, 20 min). The membrane was blocked with 5 % skim milk in PBS at 4 °C overnight and incubated with a 1/5000 dilution of anti-mouse IgG labelled to alkaline

Formatted: Not Highlight

phosphatase (Abcam) at room temperature for 2 hours. The membrane was washed 6 times with PBST (PBS, 0.5 % Tween-20), developed with NBT/BCIP. Recycling was conducted up to five times by regenerating the M13-His SPM with NaOH. After brief exposition of the M13-His SPMs to a NaOH solution, the labeled anti-phage antibody was still able to recognize the target epitope on the modified phage-SPM. This is not surprising as the whole phage structure is naturally robust toward extreme pH and temperature conditions^[21, 22, 29, 31, 37, 38]. Fe³⁺ leakage was not experimentally observed.

Formatted: Not Highlight

Electron microscopy

A 20 µL solution of magnetic particles functionalized with engineered phage was deposited onto a 300-mesh carbon-coated copper grid for 2 min. The grid was stained with a fresh 2 % uranyl acetate solution and observations were performed on a TEM (Jeol 2100, Jeol Ltd, Japan) operating at 200 keV. Environmental Scanning Electron Microscopy (FEI Quanta 3D FEG DualBeam, FEI Ltd, USA) imaging was performed at 5 keV by drying the particles and gold coating.

Acknowledgments

This work was supported by Science Foundation Ireland (grant 08/IN/2972) and the Irish National Institute for Bioprocessing Research and Training (NIBRT). Dr. Mark Platt was funded by an IEF Marie Curie fellowship (grant FP7-PEOPLE-2009-IEF 252935). The authors acknowledge Dr. Ian Reid (UCD Nano Imaging and Material Analysis Centre), Dr. Heath Bagshaw (Trinity College Dublin, Center for Microscopy), Guliano Elia (UCD Conway Institute), James O Mahony and Florence Sallas (UCD School of Chemistry and Chemical Biology) for stimulating discussions.

Competing interests

The authors have declared that no competing interests exist.

Supporting Information

Additional descriptions of the experimental procedures for magnetic particle synthesis, phage design and manipulation is available free of charge *via* the internet at www.XXX.XXX.

References

- [1] A.K. Pavlou and M.J. Belsey. *Nature Reviews Drug Discovery*, **2005**; 4, 273-274.
- [2] A.A. Shukla, J. Thommes. Recent advances in large-scale production of monoclonal antibodies and related proteins. *Trends Biotechnol*, 2010; 28(5): 253–261.
- [3] B. Kelley, G. Blank, A. Lee. Downstream processing of monoclonal antibodies: current practices and future opportunities. *In: Gottschalk U, editor. Process Scale Purification of Antibodies*. Hoboken, NJ: J Wiley & Sons; **2009**. pp. 1–23.
- [4] P. Li, A. Mahmood, G.U. Lee. *Langmuir*. **2011**; 27:6496–6503.
- [5] W.S. Chang, H. Shang, R.M. Perera, R.J. Kuhn, G.U. Lee. *Analyst*, **2008**; 133, 233-240.
- [6] Hubbuch, J.J., Matthiesen, D.B., Hobley, T.J. and Thomas, O.R.T. *Bioseparation*, **2001**; 10: 99-112.
- [7] L.M. Lacroix, D. Ho, S. Sun. *Curr Top Med Chem*. **2010**; 10:1184-97.
- [8] H. Shang, W.S. Chang, S.H. Kan, S.A. Majetich and G.U. Lee. *Langmuir*, **2006**; 22, 2516–2522.
- [9] Hubbuch, J.J. and Thomas, O.R.T. *Biotechnol. Bioeng.*, 2002; 79: 301-313.
- [10] Franzreb, M., Siemann-Herzberg, M., Hobley, T.J., and Thomas, O.R.T. ‘Product recovery by high-gradient magnetic fishing (HGFM).’ In *Process Scale Bioseparations for the Biopharmaceutical Industry*, eds. A.A. Shukla, M.R. Etzel, S. Gadam, Taylor and Francis , 2006, CRC press.
- [11] M. Sarikaya, C. Tamerler, A.K.Y. Jen, K. Schulten, F. Baneyx. *Nat. Materials*. **2003**; 2, 577–585.

Formatted: Not Highlight

Formatted: Not Highlight

- [12] J.M. Nam, C.S. Thaxton, C.A. Mirkin. *Science*. **2003**; 301:1884-6.
- [13] S.K. Lee, D.S. Yun, A.M. Belcher. *Biomacromolecules*. **2006**; 7:14-17.
- [14] C. Mao, D.J. Solis, B.D. Reiss, S.T. Kottmann, R.Y. Sweeney, A. Hayhurst, G. Georgiou, B. Iverson, A.M. Belcher. *Science*. **2004**; 303:213-7.
- [15] R. Webster. in *Phage Display: A Laboratory Manual*, eds Barbas CF, **2001**.
- [16] A.S. Khalil, J.M. Ferrer, R.R. Brau, S.T. Kottmann, C.J. Noren, M.J. Lang, A.M. Belcher. *Proceedings of the National Academy of Sciences U.S.A.*, **2007**; 104: 4892–4897.
- [17] K.T. Nam, R. Wartena, P.J. Yoo, F. Liao, Y.J. Lee, Y.M. Chiang, P. Hammond, A.M. Belcher. *Proceedings of the National Academy of Sciences U.S.A.* **2008**; 105:17227-31.
- [18] E.R. Goldman, M.P. Pazirandeh, J.M. Mauro, K.D. King, J.C. Frey, G.P. Anderson. *J. Mol. Recognit.* **2000**; 13:382–387.
- [19] S.R. Whaley, D.S. English, E.L. Hu, P.F. Barbara, A.M. Belcher. *Nature*, **2000**; 405:665–668.
- [20] V.A. Petrenko, G.P. Smith, X. Gong, T. Quinn. *Protein Eng.* **1996**; 9:797-801.
- [21] V.A. Petrenko. *Expert Opin. Drug Deliv.* **2008**; 5:825-836.
- [22] W. Noppe, F.M. Plieva, I.Y. Galaev, K. Vanhoorelbeke, B. Mattiasson, H. Deckmyn. *J Chromatogr A*. **2006**; 1101:79-85.
- [23] G.R. Souza, D.R. Christianson, F.I. Staquicini, M.G. Ozawa, E.Y. Snyder, R.L. Sidman, J.H. Miller, W. Arap, R. Pasqualini. *Proc. Natl. Acad. Sci. U.S.A.* **2006**; 103, 1215– 1220.
- [24] J.R. Newton, Y. Miao, S.L. Deutscher, T.P. Quinn. *Journal of Nuclear Medicine*. **2007**; 48:429-436.
- [25] K. Li, Y. Chen, S. Li, H.G. Nguyen, Z. Niu, S. You, CM. Mello, X. Lu, Q. Wang. *Bioconjug Chem.* **2010**; 21:1369-77.
- [26] S. Hagens, U. Blasi. *Lett Appl Microbiol.* **2003**; 37:318-23.
- [27] P. Ngweniform, G. Abbineni, B. Cao, C. Mao. *Small*. **2009**; 5:1963-9.

- [28] P.K. Jayanna, D. Bedi, J.W. Gillespie, P. DeInnocentes, T. Wang, V.P. Torchilin, R.C. Bird, V.A. Petrenko. *Nanomedicine*. **2010**; 6 :538-46.
- [29] G.P. Smith, V.A. Petrenko. Phage Display. *Chem Rev*. **1997**; 97:391-410.
- [30] A. Merzlyak, S.W. Lee. *Curr Opin Chem Biol*. **2006**; 10:246–252.
- [31] V.A. Petrenko, G.P. Smith. *Protein Eng*. **2000**; 13:589-92.
- [32] V.A. Petrenko. *Microelectron*. **2008**; 39:202–207.
- [33] D.A. Marvin, L.C. Welsh, M.F. Symmons, WR. Scott, SK. Straus. *J Mol Biol*. **2006**; 13;355:294-309.
- [34] G.P. Smith, VA. Petrenko, LJ. Matthews. *J Immunol Methods*. **1998**; 215:151-61.
- [35] A.R.M. Bradbury, S. Sidhu, S. Dübel, J. McCafferty. *Nature Biotechnology*. **2011**; 29, 245–254.
- [36] K.T. Nam, BR. Peelle, SW. Lee, AM. Belcher. *Nano Letters*, **2003**; 4 No. 1; 23-27.
- [37] R.R. Riddle, R.C. Willson, M.J. Benedik. *Biotechnology Letters*. **1999**; 21: 87–90.
- [38] M. Russel. *Mol Microbiol*. **1991**; 5:1607–1613.
- [39] J. Sambrook, E.F. Fritsch, T. Maniatis. Molecular cloning: a laboratory manual. Cold Spring Harbor, New York. **1989**.
- [40] H. Chen, X. Su, K.G. Neoh, W.S. Choe. *Anal Chem*. **2006**; 78:4872-9.
- [41] E. Gasteiger, C. Hoogland, A. Gattiker, S. Duvaud, M.R. Wilkins, R.D. Appel, A. Bairoch. □(In) John M. Walker (ed): *The Proteomics Protocols Handbook*, Humana Press. **2005**; 571-607. <http://us.expasy.org/tools/protparam.html>
- [42] S. Daly, T.M. Przybycien, R.D. Tilton. *Langmuir*. **2003**; 19, 3848.
- [43] T.I. Samoylova, N.R. Cox, N.E. Morrison, L.P. Globa, V. Romanov, H.J. Baker, V.A. Petrenko. *BioTechniques*. **2004**; 37:254-260.
- [44] K.N. Avery, J.E. Schaak, R.E. Schaak. *Chem. Mater*. **2009**; 21, 2176-2178.

- [45] J. Milton Harris. Poly(ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications, *Plenum Publishing*, New York, **1992**; 1-13.
- [46] V. Platt, Z. Huang, L. Cao, M. Tiffany, K. Riviere and F.C. Szoka, Jr. *Bioconjugate Chem.* **2010**; 21, 892–902.

Received: ((will be filled in by the editorial staff))
Revised: ((will be filled in by the editorial staff))
Published online on ((will be filled in by the editorial staff))

Figure Captions.

Table Of Contents Figure: Rendered images illustrating the M13 bacteriophage assembled on superparamagnetic beads through the pIII proteins on the end of the virion or the pVIII proteins on the sides of the virion.



Figure 1 Computer rendered models of the M13 bacteriophage coat proteins reconstructed from the crystal structures of the individual proteins. a) Schematic of the M13 phage in which

the individual coat proteins have been assembled in a filamentous structure on DNA. A silica (M13Si) or hexahistidine (M13His) binding sequence was genetically fused to the N-terminal of the pIII coat protein (colored red). b) Top and side-views of the M13 pVIII protein assembly along the central axis of the phage with DNA removed. The distance between two neighboring pVIII units is approximately 2.7 nm. c) A single subunit structure of coat protein pVIII is shown as a ribbon diagram (magenta: DNA-binding segment, grey: hydrophobic segment and orange: effective N-terminal domain) with the reactive residues highlighted, of which Lys-8 is easily accessible on the phage surface. Chemical modifications can occur on the ~2700 copies of pVIII on the whole phage surface (~ 99 % of the phage protein content).

Formatted: Not Highlight

Figure 2 Characterization by MALDI-TOF mass spectroscopy of wild and chemically modified recombinant M13-His bacteriophages: a) wild-type-pVIII, b) biotinylated-pVIII, c) double-biotinylated-pVIII, d) Cy5-pVIII, e) PEG₂₅₀₀-pVIII, and f) Cy5-PEG₂₅₀₀-pVIII.

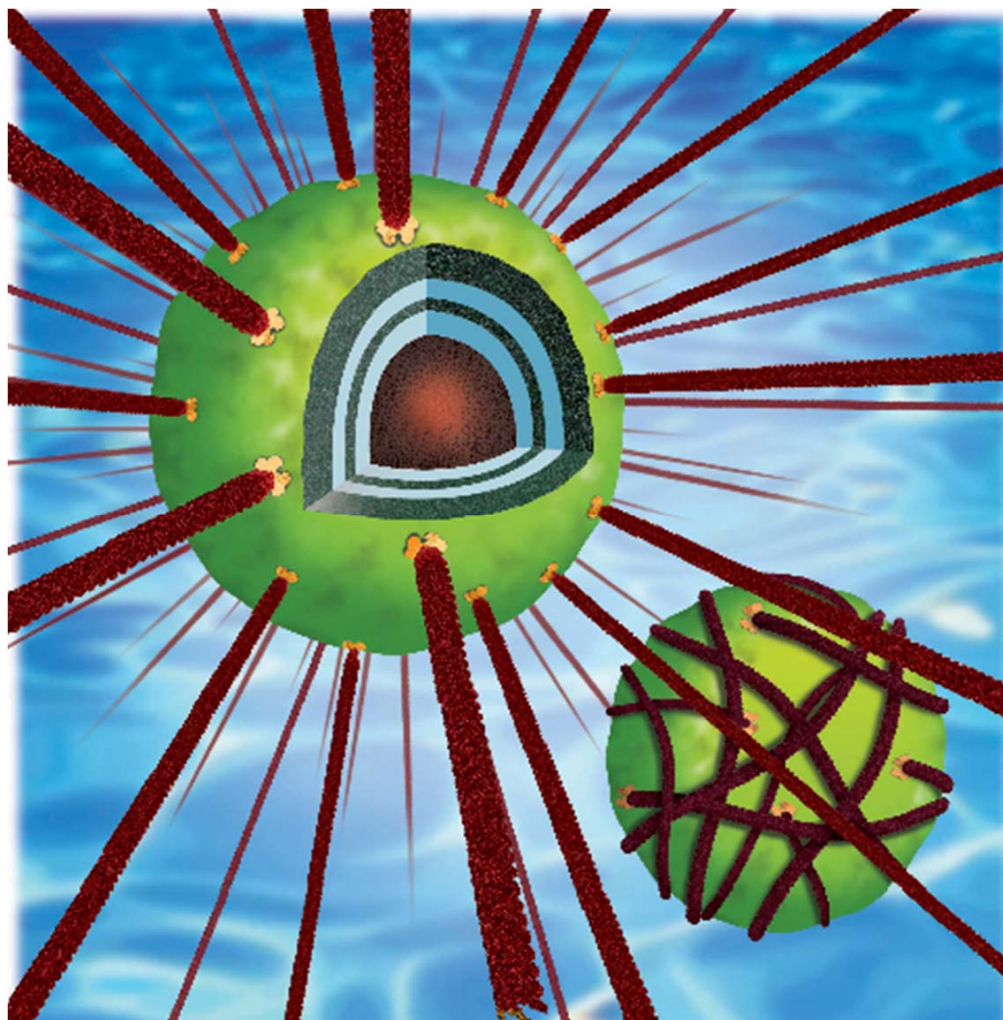
Figure 3 Characterization of phage binding to SPMs using pIII and pVIII surface coat proteins. a) Saturation phage grafting densities on SPMs measured by flow cytometry. Immobilized bacteriophages onto magnetic particles were detected using anti-pVIII-FITC antibodies. Results are displayed as number of phages per particle.

b) Measurement of M13 binding to SPMs as a function of phage concentration using a solid-phase immunoassay. The colorimetric assay was performed with mouse anti-pVIII antibodies. Increasing amounts of SPMs functionalized with M13 were incubated with anti-pVIII phage antibody coupled to HRP. After washing, peroxidase activity onto magnetic particles was measured by TMB detection at 450 nm and compared to the titration curve performed using M13 immobilized on a 96-well plate. c) Immunoblot of the affinity purified fraction. 1: mouse IgG control, 2: eluted fraction pH 2.2 (M13His) and 3: final wash fraction (before elution).

Formatted: Not Highlight

Figure 4 Electron microscope images of SPMs and phage-SPM complexes. a) SEM image of bare SPM. b) SEM image of M13 bacteriophage covalently immobilized through the pVIII coat protein on SPMs with the NHS chemistry. c) and d) FE-SEM images illustrating the end-on assembly of genetically engineered M13His virus on NTA functionalized SPMs. e) TEM image acquired using negative staining illustrating the end-on assembly of wild-M13 virus on anti-pIII functionalized SPMs. f) TEM images of M13biot bound to neutravidin coated SPM. The filamentous structures of M13 are clearly visible on the surface of the SPMs.

Proposed Cover Figure. Depiction of the M13 bacteriophage virions immobilized on the superparamagnetic beads either through the pIII proteins on the end of the phage or the pVIII protein on the side of the phage. These two immobilization schemes result in an end-on phage-superparamagnetic bead assembly or a monolayer of phage covering the superparamagnetic beads in a side-on orientation, respectively.



Supplementary Section

This supplement contains Materials and Methods, Figures S1 to S3, and References.

Phage precipitation

A single phage plaque was added to a 10 mL of 2xYT containing mid-log *E.coli* ER2738 and 10 µg/mL of tetracycline. After incubation at 37 °C for 4 hrs, the culture was centrifuged at 6000 g, at 4 °C for 15 min. Phage was precipitated from supernatant with 1/5 volume of PEG buffer (20 % PEG 8000, 2.5 N NaCl). The mixture was kept at 4 °C overnight and centrifuged at 10,000 g for 10 min at 4 °C. The phage pellet was dissolved in 500 µl of PBS, pH 7.4, centrifuged at 20,000 g for 10 min at 4 °C to remove the remaining cell debris, and stored at 4 °C. The concentration was evaluated spectrophotometrically using an extinction coefficient of 3.84 cm²/mg at 269 nm ^[1, 2].

Fluorescent labelling

M13 (10¹¹ pfu/mL) was added to one vial of the mono-reactive Cy5 dye (GE Healthcare, Dublin, Ireland). The reactive NHS-ester covalently attached to aliphatic amino groups on the phage surface. After incubation on the orbital shaker at room temperature in the dark for 1 h, the unreacting reagents were removed by dialysis and the labelled phages were further purified. Fractions were collected as monitored by a UV detector (at 269 nm); the first fraction contained labelled phages while subsequent peaks contained unbound fluorophore. Labelled phages were stored in the dark at 4°C until use.

Magnetic particles synthesis and characterization

Reagents

Ferrous chloride, ferric chloride, trifluoroacetic acid, TFA, ethanol, perchloric acid, Sodium dodecylsulphate, SDS, Hexanes, polyethylenimine 50 wt % (Mw 1300 and Mw 60K, potassium carbonate, 2-(N-morpholino)ethanesulfonic acid, 4-morpholineethanesulfonic acid, MES, polyacrylicmaleic acid, PAAMA, potassium hydroxide, sulphuric acid, tetraethylortho

silicate, TEOS, $N_{\omega}N_{\alpha}$ -bis(carboxymethyl)-L-lysine hydrate, dextrane, were all purchased from Sigma Alrich. Ammonium hydroxide and oleic acid were purchased from Fisher Scientific. N -hydroxysulfosuccinimide, sulfo-NHS and, 1-Ethyl-3-[3 dimethylaminopropyl]carbodiimide hydrochloride, EDC. BOC-NH-PEG-NHS (Mw 3000), and CH_3O -PEG-NHS (Mw 2000) were purchased from RAPP Polymere, Germany. Sulfosuccinimidyl 4-[N -maleimidomethyl]cyclohexane-1-carboxylate, Methyl N -succinimidyl adipate, MSA, was purchased from Thermo Scientific. Neutravidin was purchased from Invitrogen. All chemicals and proteins were used as purchased without further purification. Buffers used were 50 mM MES buffer pH 6.0, 50 mM carbonate buffer pH 8.2, Phosphate buffered saline, PBS (2.7 mM KCl, 0.137 M NaCl and 0.01 M phosphate) pH 9.0. For zeta potential measurement, a 1 mM potassium sulphate solution was brought to pH 4, 6, 8 or 10 using potassium hydroxide or sulphuric acid.

Hydrophobic ferrofluid synthesis

A detailed outline of the Iron oxide synthesis can be found elsewhere (Shang, 2006), briefly; 48 g $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and 98 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were dissolved in 250 mL deoxygenated water in a one-litre three neck flask under a N_2 atmosphere. The flask was placed into an ice bath whilst ammonium hydroxide (200 mL) was added rapidly with vigorous stirring. The solution was kept at 0 °C for 45 min, then the solution was heated to 85 °C for one hour, before 30 mL of oleic acid was added and heating was continued for a further 60 mins. The flask was allowed to cool to room temperature before being transferred to a 600 mL beaker. A magnet was placed next to the beaker to collect the black precipitate, which was washed three times with ethanol (200 mL). After each wash, a magnet was placed next to the beaker and the ethanol solution poured to waste. This process was repeated with DI water (200 mL) three times followed by 20 % perchloric acid (200 mL) three times, DI water (200 mL) three times and finally ethanol (200 mL) three times. After the last ethanol wash, the solution was discarded

and hexane (400 mL) was added to the beaker. The black precipitate (iron oxide nanoparticles) was dispersed in hexane resulting in a non-aqueous ferrofluid.

Micron sized carboxyl superparamagnetic beads synthesis

The hexane ferrofluid was diluted to a concentration of 15 mg/mL (wt/wt %) of Fe₃O₄. The ferrofluid was mixed with a dextran 25 % solution containing 2 % SDS (wt/wt %) to create a crude emulsion, after which it was passed through a shear device at 1 mL/min, with a distance between the inner and outer shear wall of 150 mm. The resultant emulsion was collected in a bath containing 2 % SDS and the hexane was allowed to evaporate over night creating micron sized aggregates of iron oxide nanoparticles. These were then submitted to a carboxyl surface chemistry by first incubating them in a 10 wt % PEI (molecular weight 75 K) for 16 hours and then incubating them in a 10 wt % PAAMA solution for 5 hours. After each incubation stage, the aggregates were washed three times with DI water.

NHS activated beads

NHS activation – 1 mL of carboxyl coated beads (5 mg/mL) were placed into a 1.5 mL centrifuge tube and washed 3 times with MES buffer before being resuspended in 0.5 mL of MES buffer. The beads were then placed into a sonic bath for 2 min, before a solution of 5 mg EDC, and 5 mg sulfo-NHS, in 0.5 mL MES buffer, was added. The solution was stirred rigorously and placed again into the sonicate bath for 30 sec. The beads were incubated at room temperature on a rotating wheel for 30 mins, before being washed with MES buffer (1 mL) twice. This procedure yielded NHS activated beads.

Coupling Neutravidin to carboxyl beads

The same procedure to prepare NHS activated beads was used, after which, the beads were incubated with 1 mL neutravidin solution (5 mg/mL) in MES buffer and incubated at room temperature for 2 hours on a rotating wheel. To ensure unreacted NHS groups did not remain

after 2 hours the protein solution was removed and the beads were incubated in a glycine solution pH 8 for 20 mins, then washed with PBS three times and stored at 4 °C until used.

Silica coating of carboxyl beads

500 µL of beads (10 mg/mL) were added to 5 mL of ethanol, and 10 mL of TEOS in a 15 mL falcon tube and stirred at room temperature for 5 mins. Then, 120 mL of ammonium hydroxide solution was added with continuous stirring. After 6 hours the beads were collected with a magnet and washed 3 times with DI water, before being resuspended in 10 mL of DI water and placed in sonic bath for 10 mins and subsequently, washed with water and stored in DI at 4°C. This process typically coated the beads with a silica shell of approx 60 nm, confirmed by EDAX and TEM imaging (not shown here).

Surface modification with amine groups

The same procedure to prepare NHS activated beads was used, after which 1 mL of 5 % PEI (MW 1300) solution in carbonate buffer. The particles were sonicated for 5 mins before placing them on a rotating wheel at room temperature for 2 hours. They were washed three times with carbonate buffer before being resuspended in the buffer of choice (typically carbonate buffer) to allow continuation onto the PEG coating stage.

Surface modification with Polyethyleneglycol (PEG)

Amine modified beads were prepared fresh for each PEG modification using the procedure described above, following the last wash stage in the amine modification step the solution was removed. At this stage, 1 mL of PEG solution (5 mg BOC-NH-PEG-NHS and 15 mg mPEG-NHS in carbonate buffer (as described above) and 0.6 M K₂SO₄) was added. The beads were sonicated for 5 mins before incubating them on rotation wheel for 2 h at 50°C. Finally, the particles were washed 3 times with carbonate buffer and sonicated briefly after each wash. These particles can be stored in buffer or DI water at 4 °C for up to 2 months.

Coupling NTA to PEG

The BOC group protecting the amine attached to the PEG was removed by washing the beads with a 50 % TFA solution for 4 mins. After, the beads were washed with PBS three times and resuspended with 1x PBS. 1 mL of MSA solution (2 mg/mL) was added to the particles, after incubating the beads with the MSA solution for 2 hours the beads were washed with a PBS pH 9.5 solution for 16 hours. This resulted in the amine terminated PEG groups being converted to carboxyl groups. At this point the same procedure to activate the beads with NHS was used as described above. This yielded a PEG coated particle with carboxyl group activated with NHS. The ratio of carboxyl group to methoxy groups was kept constant at 1:3. The NHS activated beads were then added to a 1 mL PBS solution containing NTA at 10 mg/mL for 2 hours before being washed with PBS and subsequently stored in PBS (0.05 % tween).

Zeta potential measurements

All zeta potential measurements were performed using a Malvern Zetasizer Nano ZS, using the DTS-1060 cells. 20 mL of particles were placed into a 1.5 mL centrifuge tube and washed three times with 1 mL of the required buffer before being resuspended in appropriate buffer and pH.

Reagents, bioinformatics and data analysis

Streptavidin-horseradish peroxidase polymer conjugate was obtained from Sigma-Aldrich Ireland; FITC anti-pVIII antibody from Abcam Laboratories (Cambridge, UK). Anti-pVIII-HRP monoclonal antibody was purchased from GE Healthcare Ltd. (Buckinghamshire, UK). This mouse antibody reacts specifically with the bacteriophage M13 major coat protein product of gene VIII. Mouse anti-pIII monoclonal antibody was obtained from NEB (ISIS Ltd, Bray, Ireland): it targets C-terminal residues on M13 pIII. All other reagents were purchased from Sigma-Aldrich Ireland Ltd (Dublin, Ireland) of molecular biology research grade. Phage

solutions and buffers were filtered before use through 0.45 μm and 0.22 μm membranes respectively. Neodymium permanent magnet was used according to the manufacturer's instructions ^[3]. Protein Data Bank entries 1IFJ and 1G3P were used as templates for M13 structure reconstitution. Structural observations of M13 proteins and picture renderings were made using PyMol Molecular Graphics System ^[4].

Supplementary figures

a)

KpnI NcoI EagI

G GTA CCT TTC TAT TCT CAC TCG CAC CAT CAC CAT CAC CAT GGC GGA GGG TCC TCG GCC G
V P F Y S H S H H H H H H G G G S S A
C CAT GGA AAG ATA AGA GTG AGC GTG GTA GTG GTA GTG CCG CCT CCC AGG AGC CGG C

10 20 30 40 50

b)

KpnI EagI
 | |
 G GTA CCT TTC TAT TCT CAC TCT TGT CAC AAG AAA CCG AGC AAG AGC TGC GGC GGA GGG TCC TCG GCC G
 V P F Y S H S C H K K P S K S C G G G S S A
 C CAT GGA AAG ATA AGA GTG AGA ACA GTG TTC TTT GGC TCG TTC TCG ACG CCG CCT CCC AGG AGC CGG C
 10 20 30 40 50 60

Figure S1 Map and sequences of the designed M13 protein pIII mutants (M13His) (a) and M13Si) (b). Cloning has been performed into M13KE (NEB) between KpnI and EagI. The NcoI restriction site was used for fast screening and capillary electrophoresis DNA sequencing (Applied Biosystems) of the final constructs were performed using ~96gIII primer (5'-CCC TCA TAG TTA GCG TAA CG- 3').

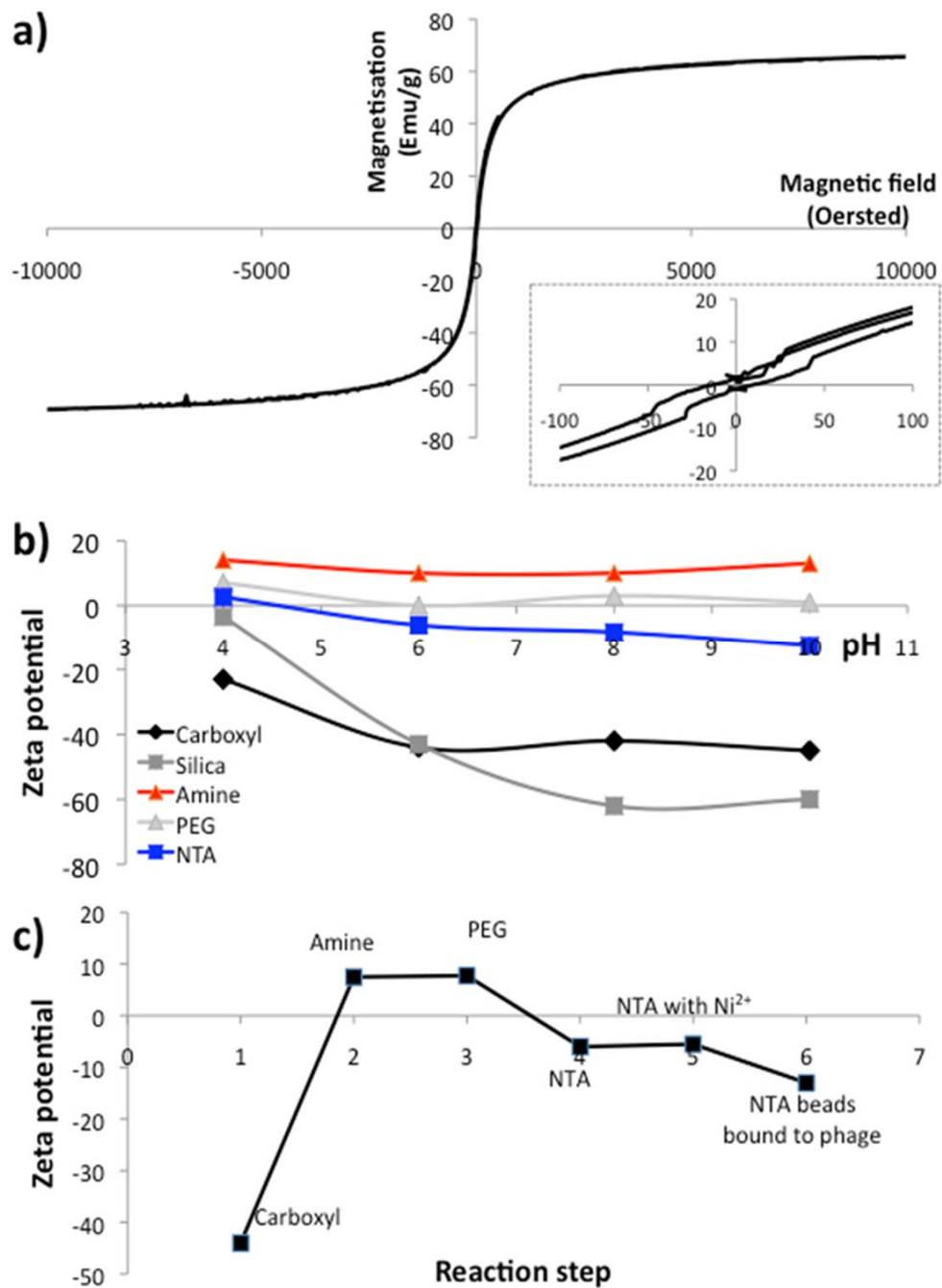


Figure S2: a) Magnetization curve of the particles used in this study. b) Variation of zeta potential against pH for the various beads and their surface chemistries. The carboxyl beads are predominantly negative across the pH tested. Amine beads appear positive, and the PEG coated beads and NTA coated beads are close to zero zeta potential across pH ranges 4-10. c) The zeta potential taken at pH 6 for the beads during each preparation stage. They start negative with a carboxyl coating, before moving to a positive zeta potential with an amine layer. The PEG layer appears to have little effect upon the zeta potential, but converting the amine to a NTA group shifts the zeta potential to a negative value. By mixing the beads with Ni and then phage the zeta potential shifts to a larger negative value.

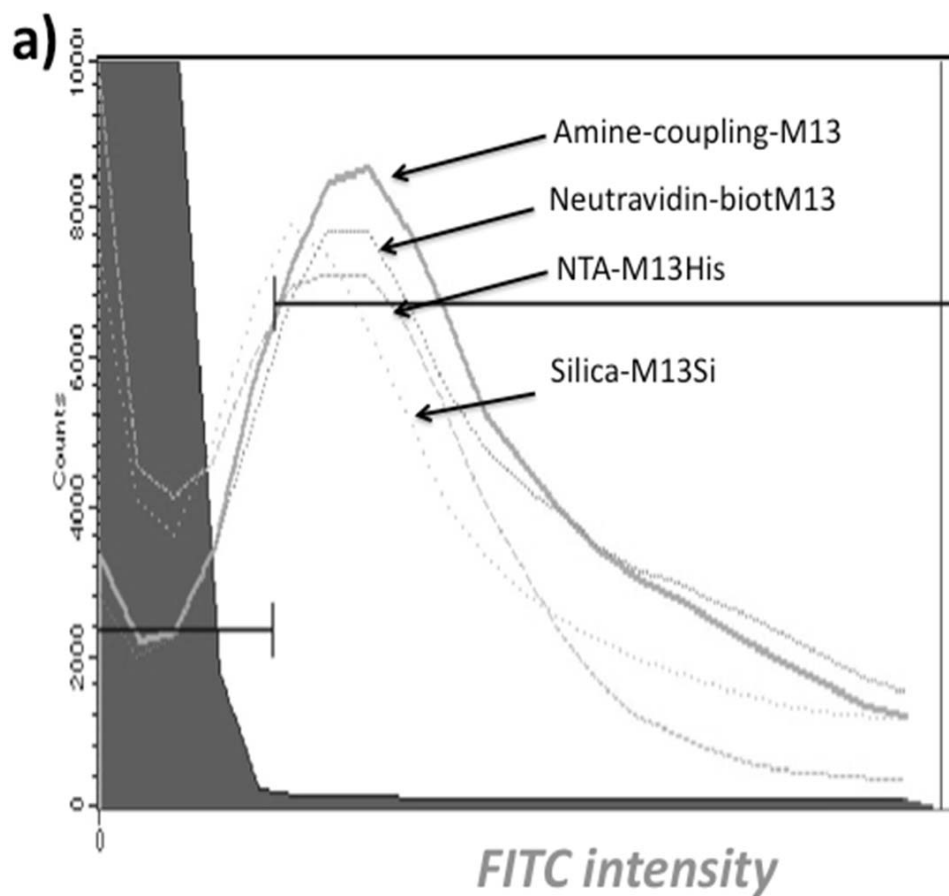


Figure S3: a) Overlay of fluorescence histograms from M13-linked magnetic particles obtained by FACS using anti-pVIII-FITC antibody.

References

- [1] K. Zimmermann, H. Hagedorn, C.C. Heuck, M. Hinrichsen, H. Ludwig. *The Journal of Biological Chemistry*. **1986**; 261, 1653-1655.
- [2] S.A. Berkowitz, L.A. Day. *J Mol Biol*. **1976**; 102:531-47.
- [3] Dynal, Invitrogen Inc. www.invitrogen.com
- [4] W.L. DeLano. *The PyMOL Molecular Graphics System*. **2002**, DeLano Scientific, San Carlos, CA, USA.