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Research Article**Magnetizable antibody-like proteins**

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6 Abbreviations: AMPSO N-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-
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8 hydroxypropanesulfonic acid, cDNA Complimentary DNA, EDX Energy
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10 dispersive X-ray, H Heavy, L Light, scFv Single Chain Fv, SEM Scanning
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12 electron microscopy, SPR Surface plasmon resonance, TEM Transmission
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14 electron microscopy, TMO Trimethylamine-N-oxide.
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For Peer Review

Abstract

Use of paramagnetic particles to isolate molecules or cells from complex media is well established. Typically, particles are manufactured and coated with a biological molecule which confers specific biorecognition. Incubation of particles with sample and exposure to magnetic fields isolates the species of interest. We designed, produced and assessed magnetized fusion proteins consisting of the antigen binding portion of an antibody (single chain Fv; scFv) fused to the heavy chain of the iron binding protein ferritin. The fusion protein subunits expressed in *E. coli* assemble to form a fusion protein consisting of a ferritin sphere with scFvs on the surface. The fusion proteins were chemically magnetized by introducing a paramagnetic iron core. The resultant fusion protein was shown to be magnetizable as well as capable of binding target antigens. These “organic” magnetizable particles possess a number of theoretical advantages over traditional inorganic particles.

1. Introduction

Inorganic magnetic particles chemically coated with biological molecules (particularly antibodies) have been used since the 1970s for amongst other applications: *in vitro* analysis[1], cell labeling and separation[2] and nucleic acid isolation[3] and *in vivo* uses such as diagnosis and therapy[4]. The routine use of magnetic particles in research and clinical diagnostics is well established. These particles are often formed from a super-paramagnetic iron core with a polymer coating to which agents can be conjugated via a number of chemical attachment methods[5]. Examples of biological molecules attached to magnetic particles include antibodies[6], nucleic acids[7, 8], peptides[9], carbohydrates and other polymers[10].

Although inorganic magnetic particles are widely used, issues including variability in coating efficiency invariably occur. Coating inorganic particles with biological molecules is generally a random process, both in terms of the number and orientation of molecules associated with a given particle. For example, when conjugating antibodies such as IgG molecules to a particle, it is unlikely that each and every antibody molecule on that particle will have both antigen recognition sites orientated in an optimal manner, away from the particle and facing the milieu. In addition, each particle is likely to have varying numbers of antibodies on its surface. As a result, preparations typically contain a distribution of particles with varying biorecognition functionality[11]. In applications where a limited number of particles will be used, such as in microfluidic devices or *in vivo*, this variation in number of binding sites available per particle will ultimately affect

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6 reproducibility. Chemical conjugation techniques have also been shown to
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8 decrease the functional activity of proteins[12]. The use of genetically fused
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10 recognition and paramagnetic domains on a single protein rather than chemical
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12 conjugations may overcome this.
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17 To address the variation seen when using coated inorganic microparticles, we
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19 devised a method of producing novel, bi-functional fusion proteins which have the
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21 affinity and avidity of antibodies coupled with intrinsic super-paramagnetism. The
22
23 binding specificity of the fusion protein reported in this article (depicted
24
25 diagrammatically in figure 1A) is provided via single chain variable fragments
26
27 (scFv) of antibodies and the magnetic element via the naturally occurring iron
28
29 binding protein; ferritin. The scFv elements of the fusion protein are composed of
30
31 the two globular domains involved in the binding of an antibody to its antigen,
32
33 linked by a series of small, non-polar amino acids which have been previously
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35 shown to allow scFvs to fold correctly [13, 14]. The design of the protein also
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37 provides improved avidity since multiple scFv regions can be displayed on the
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39 surface of the protein, which may decrease the dissociation rate of the fusion
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41 protein[15].
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48 Many proteins are able to store iron as an oxyhydroxide-ferric phosphate or as
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50 haem (e.g. lactoferrin and hemoglobin). However, many of these proteins do not
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52 contain many atoms of iron (or other metal) per protein molecule and may
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54 therefore exhibit low levels of paramagnetism. Proteins such as ferritin are able to
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56 store up to 4000 iron atoms within a cage-like structure.
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6 Ferritin is a large, multifunctional protein, 12-15nm in diameter, with a molecular
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8 weight of 480 kilo-Daltons (kDa). The protein consists of a cavity (6-8nm
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10 diameter) which encases iron and can also encapsulate other metals. The cavity is
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12 formed by the spontaneous assembly of 24 ferritin polypeptides folded into four
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14 helix bundles held by non-covalent bonds. Two types of subunits (heavy chain
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16 (H) and light chain (L)) form ferritin in vertebrates, each with catalytically active
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18 (H) or inactive (L) oxidase sites. The ratio of heavy and light chains can vary
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20 between ferritin isoforms [16, 17]. Jääskeläinen *et al* [18] constructed a non-
21
22 magnetic (Europium loaded) scFv:ferritin fusion protein for use in diagnostic
23
24 assays which was composed of the light chain of ferritin only and was able to
25
26 store metal ions efficiently. The ferritin used in the study reported here is
27
28 composed of both heavy and light chains and is based on one designed by Lee *et*
29
30 *al* [19] as this was reported to have increased cytoplasmic solubility when
31
32 compared to light chain alone.
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38 The iron stored within ferritin is usually in the form of hydrated iron oxide
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40 ferrihydrite ($5\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}$). Meldrum *et al.* [20] previously demonstrated that it
41
42 was possible to chemically replace the ferrihydrite core with a ferrimagnetic iron
43
44 oxide; magnetite (Fe_3O_4). This was achieved by removing endogenous iron using
45
46 thioglycolic acid to produce apoferritin. Fe(II) solution was then added under
47
48 argon with slow, controlled oxidation by the introduction of air to convert Fe to
49
50 magnetite into the apoferritin core. The Fe(II) loaded into ferritin during our study
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52 was oxidized chemically using trimethylamine-N-oxide (TMO) as described by
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54 Wong *et al* [21] for simplification and reproducibility.
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6 To construct the antibody-ferritin fusion protein for this work, the genes coding
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8 for the variable regions of antibodies were linked to the genes coding for ferritin
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10 to produce a magnetizable, antigen recognizing protein. The scFv and ferritin
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12 domains were inserted into an expression plasmid.
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18 The purpose of this study was to construct and analyze an organic, paramagnetic,
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20 antibody like molecule, similar in size or smaller than commercially available sub
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22 micrometer sized inorganic beads. By constructing the magnetic antibody like
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24 protein genetically rather than chemically, the exact number of binding molecules,
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26 their orientation and function can be controlled.
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30 31 32 2. Materials and Methods

33 34 Protein expression, purification and analysis

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36 Human ferritin heavy and light chain genes were amplified from a human liver
37
38 cDNA library. PCR products were ligated using overlapping PCR. The full length
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40 heavy (H) and light (L) chains (1074 base pairs) were ligated into a pGEMTeasy
41
42 plasmid (Promega) for sequencing. PCR amplifications of a murine monoclonal
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44 scFv directed against fibronectin and the full length ferritin heavy and light chain
45
46 single chain were conjugated using overlapping PCR. The overlap product was
47
48 ligated into the pRSET vector (Invitrogen) and sequenced to confirm mutations
49
50 had not been introduced. The ferritin fusion protein was purified via the
51
52 incorporated N-terminal hexahistidine tag using Immobilised Metal Ion
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54 Chromatography (IMAC). Competent *E.coli* cells were transformed with the
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6 vector and cultured until reaching mid-log phase, approximately 3 hours (A_{600} 0.6
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8 at 37°C). Expression was induced by the addition of IPTG (1 mM) and cells
9
10 propagated for a further 4 hours before harvesting. The cell pellet was re-
11
12 suspended and lysed in denaturation buffer (8 M Urea, 50 mM NaH_2PO_4 , 300 mM
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14 NaCl, 5 mM Imidazole, pH 8.0) then left to mix overnight on a rotor. The crude,
15
16 denatured lysate was clarified by centrifugation (6,000 rpm, 60 min at 4°C). The
17
18 resulting supernatant was filtered through 0.8 μM gyrodisc filters (Orange
19
20 Scientific) before being applied to an IMAC column charged with Ni^{2+} ions
21
22 (HiTrap 1ml; GE Healthcare). Non-specifically bound material was washed from
23
24 the column using IMAC wash buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 5 mM
25
26 Imidazole, pH 8.0) followed by on-column refolding by reduction of the urea
27
28 from 8 to 0 M over 20 column volumes. The refolded material was then washed
29
30 with a further 20 column volumes of IMAC wash buffer. Elution of the bound
31
32 material was achieved via gradient using IMAC wash Buffer and IMAC elution
33
34 buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 500 mM Imidazole, pH 8.0) from 0-
35
36 100% IMAC elution buffer over 30 column volumes. Protein fractions (1mL)
37
38 were collected and analyzed by SDS PAGE gel (Criterion 4-20% Tris/Glycine)
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40 (see supplementary information figure S2).
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51 The design of the scFv:ferritin fusion construct incorporates the scFv at the amino
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53 terminus of the ferritin H chain (to ensure expression on the surface of ferritin as
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55 shown by Ahn *et al* [22]) which is in turn, fused to the amino terminus of the
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6 ferritin L chain (to increase the solubility of the fusion protein in *E. coli* as shown
7
8 by Lee *et al* [19]).

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10 The amino acid sequence of the anti-fibronectin scFv:ferritin fusion peptide can
11
12 be seen in figure 2. This fusion peptide is predicted to have 611 amino acids and a
13
14 molecular weight of 67,731 Daltons (x12 subunits = 813kDa for the assembled
15
16 protein).

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22 Binding kinetics of the anti fibronectin scFv:ferritin fusion protein were assessed
23
24 by SPR using a SensiQ 100 instrument (Icx Nomadics). $1\mu\text{g mL}^{-1}$ fibronectin
25
26 peptide (amino acids 16-42) was coupled to a SensiQ carboxylic acid chip using
27
28 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and N-
29
30 hydroxysuccinimide. Varying dilutions of anti-fibronectin scFv:ferritin fusion
31
32 protein were flowed over the tethered peptide. Sensorgram data was analyzed
33
34 using SensiQ Qdat analysis software to calculate kinetic parameters (kinetic
35
36 association constant; k_a and kinetic dissociation constant; k_d).

43 Fusion protein magnetization and purification

44
45 To magnetize the fusion protein, endogenous (non-magnetic) iron was removed
46
47 from the ferritin core to produce scFv:apoferritin. The scFv:ferritin (amounts
48
49 varied between 1mg and 7mg) was dialyzed for 15 minutes against 800mL 0.1M
50
51 sodium acetate buffer pH4.5 (pre-sparged with N_2). 2mL thioglycolic acid (70%
52
53 w/w) was added to the sodium acetate buffer. ScFv:ferritin was dialyzed against
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55 this solution for 2 hours, after which a further 1mL thioglycolic acid was added to
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6 the sodium acetate buffer. The scFv:ferritin was then dialyzed against this solution
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8 for an additional hour. The scFv:ferritin was then dialyzed against fresh 3mL
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10 thioglycolic acid in 800mL 0.1M sodium acetate buffer pH4.5 for 1 hour (N₂
11
12 sparging maintained throughout dialysis).

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15 The scFv:apoferritin was dialyzed against 2 litres phosphate buffered saline (PBS;
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17 9.6mM Na₂HPO₄, 1.5mM KH₂PO₄, 2.7mM KCl, 0.137M NaCl; pH7.4) with
18
19 stirring at room temperature for 1 hour. The scFv:apoferritin was then dialyzed
20
21 against 3L PBS at 4°C overnight with stirring.
22
23

24
25 To re-mineralize the fusion protein, the scFv:apoferritin was dialyzed against 1L
26
27 0.05M AMPSO (N-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-
28
29 hydroxypropanesulfonic acid) buffer, pH8.6 for 15 minutes at room temperature.

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31 A N₂ sparge was introduced and the dialysis continued for a further 2 hours under
32
33 continual N₂ sparging. To remove contaminating trimethylamine, trimethylamine-
34
35 N-oxide (TMO) was heated to 80°C followed by cooling to room temperature
36
37 prior to use. TMO (0.07M) and iron (II) ammonium sulphate (0.1M) solutions
38
39 were used for re-mineralization. The scFv:apoferritin (e.g. 1mg in 10mL) was
40
41 transferred to a 25mL round bottomed flask, and placed into a water bath at 60°C.
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45 The flask was attached to a N₂ gas inlet after contents had reached thermal
46
47 equilibrium. The solution was sparged for 15 minutes after which a N₂ blanket
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49 was maintained on the surface throughout subsequent additions of iron (II)
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51 ammonium sulphate and TMO. Additions of 100mM iron (II) ammonium
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53 sulphate (108µl) and 70mM TMO (72µl) were made as listed below and were
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55 based on the findings of Wong *et al.* [21]. Iron and TMO were incrementally
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6 added at a stoichiometric ratio of 3:2. The iron (II) ammonium sulphate solution
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8 was continuously sparged with N₂ throughout the experiment to avoid oxidation.
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10 18µl iron (II) ammonium sulphate was added to the scFv:apoferritin. After 25
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12 minutes, 15µl iron (II) ammonium sulphate and 10µl TMO were added. After 15
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14 minutes, 30µl iron (II) ammonium sulphate and 20µl TMO were added. Three
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16 subsequent additions of 15µl iron (II) ammonium sulphate and 10µl TMO were
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18 added to the solution at 15 minute intervals. Finally, 10µl TMO was added at the
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20 end of the mineralization process. The solution was prone to evaporation during
21
22 the experiment due to the high temperatures and low volumes involved; therefore
23
24 approximately 15mL AMPSO buffer was added during the experiment to
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26 maintain reaction volume. The re-mineralized fusion protein was dialyzed against
27
28 PBS overnight. The volumes of iron (II) ammonium sulphate and TMO were
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30 adjusted for the amount of protein in subsequent experiments.
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36 The magnetized fusion protein was concentrated using a sterile magnetic cell
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38 concentrator (MACS LS column; Miltenyi Biotec, Gladbach, Germany). The
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40 column was placed in a magnetic separator and pre-equilibrated with 3mL PBS.
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42 The magnetized scFv:ferritin fusion protein solution was added to the column and
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44 allowed to flow through under gravity. The column was removed from the magnet
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46 and the retained protein eluted from the column using the sterile plunger supplied.
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51 52 53 Binding and iron content analysis

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55 To determine whether the magnetized fusion protein retained binding affinity for
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57 fibronectin, ELISA analyses were performed. Full length human fibronectin
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6 (Haematologic Technologies Inc. Cambridge Bioscience Ltd, UK) or fibronectin
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8 peptide (amino acids 16-42, Fusion Antibodies Ltd.) were diluted to $20\mu\text{g mL}^{-1}$ in
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10 carbonate buffer (0.015M sodium carbonate, 0.036M sodium hydrogen carbonate
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12 pH9.6). $100\mu\text{l}$ was used to coat wells of a microtitre plate and incubated overnight
13
14 at 4°C . Coating solutions were removed from the plate and washed 3 times using
15
16 PBS. $100\mu\text{l}$ of $10\mu\text{g mL}^{-1}$ scFv:ferritin (not magnetized) in PBS was added in
17
18 duplicate to the coated wells as a positive control. Wells containing no fusion
19
20 protein were used as a background control. The flow-through solution,
21
22 magnetized MACS column concentrated scFv:ferritin and PBS (negative control)
23
24 were added undiluted to other wells in duplicate. The plate was incubated at 20°C
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26 for 1 hour before washing as before. Murine anti-human ferritin antibody was
27
28 diluted to $10\mu\text{g mL}^{-1}$ in PBS and added to all wells and incubated at room
29
30 temperature for 1 hour. The plate was washed as before. Alkaline phosphatase
31
32 conjugated goat anti-mouse polyclonal antibody was diluted 1/1000 in PBS and
33
34 $100\mu\text{l}$ added to each well, then incubated at room temperature for 30 minutes. The
35
36 solution was removed and wells washed as before. $100\mu\text{l}$ per well substrate
37
38 (Phenolphthalein phosphate) was added to wells for 1 hour. $100\mu\text{l}$ of strongly
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40 alkaline stop solution was added to all wells. Absorbances (550nm) were recorded
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42 using a plate reader.
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53 Experiments were performed to ascertain if the magnetized fusion protein could
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55 bind to activated platelets which express fibronectin on their surface using
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57 microscopic analysis. Blood was collected into an EDTA vacutainer and cells
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6 pelleted at 600g for 20 minutes. The platelet rich supernatant was removed and
7
8 exposed to air for 30 minutes to activate the platelets. 2 μ L was mixed with 50 μ L
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10 magnetized or non-magnetized (10 μ g/mL) fusion protein or 50 μ L human ferritin
11
12 (10 μ g/mL Sigma F6879) or 50 μ L PBS and transferred to a chambered microscope
13
14 slide and incubated for 30 minutes at room temperature to allow platelets to settle.
15
16 100 μ L of a 3:1 methanol:acetic acid mixture was slowly added to the chamber
17
18 and incubated for 30 minutes at room temperature. The solution was gently
19
20 removed and 100 μ L Giemsa stain (diluted 1:10 in methanol) added to the
21
22 chamber. This was incubated for 15 minutes at room temperature before being
23
24 removed. The chamber was rinsed with 100 μ L methanol and then 100 μ L reverse
25
26 osmosis water (RO water). The water and chamber walls were removed and the
27
28 slide was left to dry at 37°C for 30 minutes before visual inspection at 400x
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30 magnification.
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39 Environmental scanning electron microscopy (Philips XL 30 - ESEM) fitted with
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41 an energy-dispersive x-ray (EDX) module (Oxford Instruments) was used to
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43 determine the iron content of the fusion protein. Samples of magnetized
44
45 scFv:ferritin, unmodified scFv:ferritin and apo scFv:ferritin were diluted 1:1 with
46
47 0.01M zinc chloride and 5 μ L of the mixture dried on to a glass cover-slip. Three
48
49 low power images of each sample, taken at different points, were analyzed by the
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51 EDX module and the ratio of the Fe peak at 6.404 keV and the Zn peak at 8.639
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53 keV calculated (Figure 5).
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6 Transmission electron microscope (Philips CM10 - TEM) pictures were obtained
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8 by drying a 5 μ L sample of the magnetized fusion protein, diluted 1/1000 in
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10 physiological saline, on a carbon-formvar coated grid. Pictures were taken at
11
12 various magnifications where the proteins imaged were suitably dispersed.
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14

15 Scanning electron microscopy (SEM) with energy-dispersive x-ray (EDX) was
16
17 used to determine the iron content of the fusion protein (figure 4). Samples were
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19 spiked with 0.01M zinc chloride and the ratio of Fe to Zn compared.
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3. Results

Purification of scFv:ferritin fusion protein

The genes for a murine anti-fibronectin scFv and human ferritin were ligated into a plasmid (see supporting information figure S1). Heavy Fv and light Fv genes for a murine anti-fibronectin scFv were joined via a short linker region. Human H and L ferritin genes were ligated to the 3' end of the scFv light chain. The genes were ligated into a plasmid which was used to transform an *E. coli* strain for expression. Purification of the fusion protein from 8M urea lysed cells on nickel affinity columns did not yield intact protein. Therefore in subsequent productions, induced, over-expressed fusion protein was obtained from isolated inclusion bodies, re-solubilized and purified on nickel affinity columns (see supporting information figure S2).

Binding kinetics of the fusion protein

Binding kinetics of the anti fibronectin scFv:ferritin fusion protein were assessed by surface plasmon resonance (SPR). Resulting curves demonstrated a biphasic dissociation curve which may be due to the multiple binding regions of the fusion protein (figure 3). Analysis of the second phase of the curve (orange lines on figure 3) resulted in a k_d value of 0.00535 s^{-1} ($k_a 8.183 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) to give a dissociation constant (KD) of approximately 0.65nM which is comparable to that of monoclonal antibodies used in diagnostics and therapy.

Magnetization of the fusion protein

The fusion protein was demineralized using thioglycolic acid and sodium acetate to remove any endogeneous iron to produce apo-scFv:ferritin, prior to re-mineralization to form a paramagnetic iron core. Concentration of the magnetizable fusion protein was achieved by flowing the re-mineralized fusion protein through a magnetized column. Magnetizable material was retained, whilst non-magnetizable material (which was colorless) passed through the column. Retained magnetizable material (which was dark brown in color) could be eluted following removal of the column from the magnet (supporting information figure S3).

Analysis of scFv:ferritin antigen binding and iron content

Enzyme linked immunosorbent assays (ELISA) assays confirmed that magnetized anti fibronectin scFv:ferritin fusion protein retained binding to target antigen, both to full length human fibronectin and fibronectin peptide (amino acids 16-42) (figure 4).

Experiments were performed to ascertain if the fusion protein could bind to activated platelets which have fibronectin on the membrane surface. Microscopic analyses (figure 5) showed that both non-magnetized and magnetized fusion protein very effectively bind to platelets in comparison to a purified human ferritin control. Due to the multi-valent binding properties of the fusion protein, platelets are cross-linked in the presence of the protein. Human ferritin (used as a

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6 negative control) did not cross-link platelets. The human ferritin molecule did not
7
8 have scFv molecules on its surface indicating that the interaction of the
9
10 scFv:ferritin fusion protein with platelets expressing fibronectin was due to the
11
12 scFv portion of the fusion protein only.
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18 The re-mineralized (magnetizable) scFv:ferritin samples contained iron. This was
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20 confirmed using scanning electron microscopy (SEM) with energy-dispersive x-
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22 ray (EDX) (figure 5). Samples were spiked with zinc chloride and the ratio of Fe
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24 to Zn compared. Interestingly, EDX data indicated that scFv:ferritin isolated from
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26 inclusion bodies which had not been subject to de-mineralization by thioglycolic
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28 acid and sodium acetate, contained little or no iron. It is probable that the
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30 unfolding and re-folding process during isolation of the fusion protein resulted in
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32 demineralization.
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39 Transmission electron microscopy

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41 The diameter of commercially available inorganic paramagnetic beads typically
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43 varies between 50 nanometers and several micrometers. The scFv:ferritin fusion
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45 protein reported here was predicted to have a diameter of approximately 20nm
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47 based upon a reported diameter of ferritin of 12-15nm and a length of
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49 approximately 3nm for the scFv[22]. Images generated using transmission
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51 electron microscopy (TEM) (figure 1 B and C) supported the predicted molecule
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53 size (mean diameter of 19.5nm) and demonstrated aggregations of iron within the
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55 central cavity.
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4. Discussion

The construction and analysis of super-paramagnetic fusion proteins with the binding function of antibodies was effectively demonstrated. The binding ability was achieved using antibody fragments known as scFv and the super-paramagnetism by chemically loading the iron binding protein ferritin with magnetizable iron. The fusion protein was shown to have retained antigen binding ability using surface plasmon resonance analysis (which also demonstrated that the protein had high avidity for target antigen), ELISA and microscopic analyses of platelet binding.

For this study, an alternative scFv fusion protein was also constructed consisting of the anti-fibronectin scFv and a gene coding for human metallothionein II. This has been previously shown to be influenced by magnetic fields when endogenous zinc ions are replaced with manganese and cadmium[23]. The dissociation constant of this fusion protein (KD) was found to be 2.3nM which was 3.5 times more than that of the ferritin fusion protein (which was 0.65nM). This may be due to reduced avidity, as the SPR results showed a biphasic dissociation curve which had a fast initial dissociation followed by a much slower dissociation (results not shown). These results suggest that the scFv:metalothionein II fusion protein consists of a pair of antigen recognition sites in comparison to a scFv:ferritin fusion protein which would have 12. In addition, previous studies have shown that having linker regions of 12 amino acids or less (as in this case) forces scFv

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6 peptides to dimerise[24]. For this reason, only the ferritin fusion protein was
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8 further analyzed. A fusion protein which has reduced avidity that is able to be
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10 manipulated magnetically *in vivo* could be advantageous as it may percolate into
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12 target tissues more effectively and be cleared more rapidly than larger fusion
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14 proteins[25].
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20 The paramagnetism of the fusion protein was shown using commercially available
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22 magnetized columns (from Miltenyi Biotec) for purification and through
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24 preliminary data using a magnetometer (results not shown). In addition, other
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26 strong magnets were also found to rapidly attract the brown fusion proteins in
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28 solution (results not shown). These biological paramagnetic molecules could be
29
30 useful for a number of applications including *in vitro* diagnostic applications, *in*
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32 *in vivo* analysis (including imaging) and as therapeutic agents[26].
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39 With the advent of microfluidic and nanofluidic lab-on-a-chip based technologies
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41 for clinical diagnostic applications, it is likely that small, magnetic particles with
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43 biorecognition capability will become an integral part of some of these devices, as
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45 well as for macrofluidic applications. The very small size of magnetizable fusion
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47 proteins relative to inert, magnetic particles would confer an advantage within
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49 these devices as these particles have a relatively large surface area to volume
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51 ratio. The highly reproducible construction of the fusion proteins where a defined
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53 and constant number of binding sites can be incorporated onto a protein may also
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55 confer particular advantages in nanotechnology. In addition, these proteins are
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6 tunable; combinations of recognition moieties can easily be incorporated into the
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8 protein by genetically engineering desired antibody fragments into the protein.
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10 The advantages of this tuning includes the ability to cross-link different receptors
11 (using scFv fragments directed against these receptors) which will be of interest
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13 during *in vitro* studies. The use of simple, adaptable chemical methods to load the
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15 proteins with a paramagnetic core also confers an advantage to these proteins
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17 when compared to current methods of mass production.
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20 Other areas where magnetizable fusion proteins could have particular benefits
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22 over established inorganic based magnetizable particles include *in vivo*
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24 applications such as imaging[27,28] or therapeutics which may benefit from the
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26 use of fully human (as the scFv and ferritin proteins can be engineered to contain
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28 species specific amino acid sequences), metal containing, super-paramagnetic
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30 antigen specific biological molecules. The use of magnetizable proteins
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32 incorporating biorecognition moieties are likely to overcome some of the
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34 problems of toxicity associated with the use of micro and nanoparticles[29].
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13
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6 Figure Legends:
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8 Figure 1(A). A graphic representation of a magnetic fusion protein. The
9 scFv fragments are coloured pale blue, the ferritin subunits pale green and
10 the iron core dark red (this figure is not relative in size and is a simplified
11 representation of the envisaged fusion protein). B) Transmission electron
12 micrograph of the magnetised scFv:ferritin molecules. The average
13 diameter of the molecules is 19.5nm. Photograph C is a close-up of one of
14 the molecules. Aggregation of iron can be seen within the core.
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27 Figure 2. The amino acid sequence of the scFv-ferritin peptide is shown in
28 figure A. The linker regions joining the V_H and V_L domains of the antibody
29 sequence and the V_L of the antibody and the ferritin heavy chain are
30 highlighted in grey. The light chains of the antibody and ferritin proteins
31 are underlined. Primer sequences used to amplify and overlap the ferritin
32 and antibody genes can be seen in figure B.
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43 Figure 3. Surface plasmon resonance data for an anti-fibronectin
44 scFv:ferritin fusion protein. 1mg/ml fibronectin peptide (amino acids 16-42)
45 was coupled to a SensiQ COOH chip. Varying concentrations (0.133 μ M to
46 0.0013 μ M) of anti fibronectin scFv:ferritin fusion protein were flowed over
47 the tethered peptide. The lowest concentration is represented by the curve
48 towards the bottom of the sensorgram and the highest towards the top. As
49 the fusion protein binds to the peptide, an increase in response can be
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6 seen. When the diluted fusion protein is replaced with running buffer (blue
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8 arrow) the fusion protein disassociates from the peptide. As the curves
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10 demonstrated a biphasic distribution (which may be due to the multiple
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12 binding regions of the fusion protein) data was calculated from the slopes
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14 (shown as orange lines) after the vertical blue line.
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20 Figure 4. Evaluation of the effectiveness of using a magnet to concentrate
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22 re-mineralized scFv:ferritin fusion protein. ELISA analysis using the
23
24 binding ability of anti-fibronectin scFv:ferritin fusion protein to full length
25
26 human fibronectin (light grey) and fibronectin peptide (dark grey) was
27
28 performed. Fusion protein prior to any treatment (10 μ g/ml) (non-
29
30 magnetized scFv:ferritin); flow through material from magnetized fusion
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32 protein after passage through a magnetic column (non magnetic flow
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34 through); and fusion protein concentrated using a magnetic column
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36 (magnetized magnetic scFv:ferritin) were compared. It can be seen from
37
38 this data that magnetized scFv:ferritin is effectively concentrated using a
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40 magnet, proving that the material is indeed paramagnetic. Results are
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42 from a typical single experiment.
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50 Figure 5. Evidence for binding of fusion protein to platelets (400x
51
52 magnification). Giemsa stained platelets are pale grey in these images
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54 and aggregates of ferritin appear black. Image A is of platelets only; Image
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56 B is of platelets mixed with human ferritin; Image C is of platelets mixed
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6 with non-magnetized anti-fibronectin:ferritin fusion protein; Image D is of
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8 platelets mixed with magnetized anti-fibronectin:ferritin fusion protein. The
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10 non-magnetized and magnetized fusion proteins cross-link platelets
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12 effectively.
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18 Figure 6. SEM/EDX analysis of scFv:ferritin (100 μ g/ml original
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20 concentration) to show iron content of the fusion protein. Samples were
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22 spiked with 0.01M zinc chloride. The EDX response was normalized using
23
24 the ratio of the iron peak and zinc peak for each sample. The samples
25
26 contained: Magnetized scFv Ferritin: fusion protein that had been de-
27
28 mineralized to remove endogenous iron and re-mineralized with
29
30 paramagnetic iron; Unmodified scFv ferritin: fusion protein that had not
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32 been de-mineralized or re-mineralized; and Apo scFv ferritin: fusion
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34 protein that had been de-mineralized but not re-mineralized.
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3 Figure 1(A). A graphic representation of a magnetic fusion protein. The scFv
4 fragments are coloured pale blue, the ferritin subunits pale green and the iron core
5 dark red (this figure is not relative in size and is a simplified representation of the
6 envisaged fusion protein). B) Transmission electron micrograph of the magnetised
7 scFv:ferritin molecules. The average diameter of the molecules is 19.5nm.
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15 Photograph C is a close-up of one of the molecules. Aggregation of iron can be seen
16 within the core.
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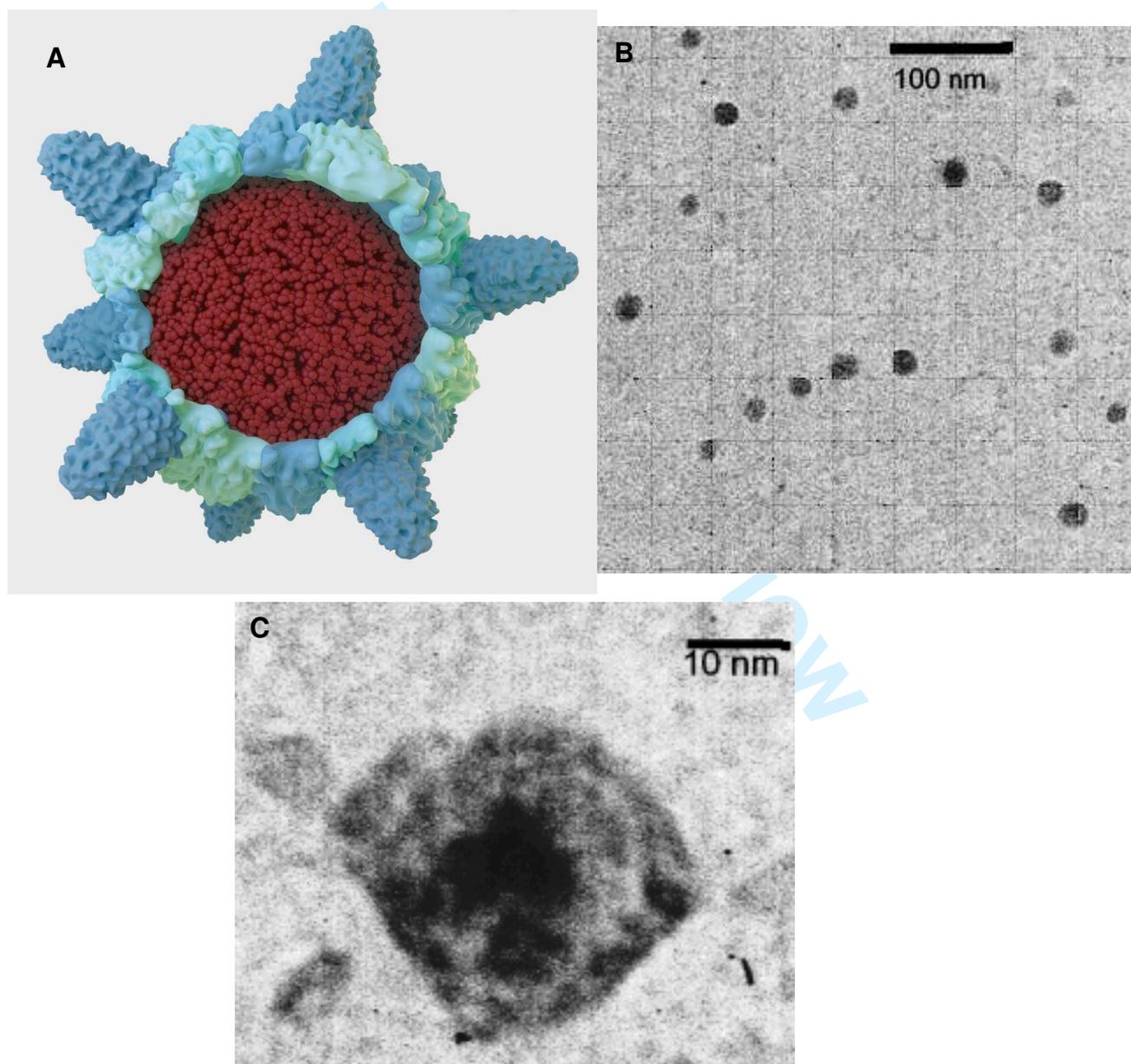


Figure 2.

A:

GSMAEVQLLES GGGLVQPGGYLRLSCAASGFTFSSFSMS
 WVRQAPGKGLEWVSSISGSSGTTYADSVKGRFTISRDN
 SKNTLYLQMNSLRAEDTAVYYCAKPFYFDYWGQGLVT
VSSGDGSSGGSGGASTGEIVLTQSPGTLSPGERATLSC
RASQSVSSSFLAWYQQKPGQAPRLLIYASSRATGIPDRF
SGSGSGTDFTLISRLEPEDFAVYYCQQTGRIPPTFGQGT
KVEIKPGSAGGSGGDSMTTASTSQVRQNYHQDSEAINR
 QINLELYASYVYLSMSYYFDRDDVALKNFAKYFLHQSHEE
 REHAEKLMKLQNQRGGRIFLQDIKKPDCDDWESGLNAME
 CALHLEKNVNQSLLELHKLATDKNDPHLCDFIETHYLNEQV
 KAIKELGDHVTNLRKMGAPESGLAEYLFDKHTLGDSDNES
MSSQIRQNYSTDVEAAVNSLVNLYLQASHTYLSLGFYFDR
DDVALEGVSHFFRELAEEKREGYERLLKMQNQRGGRALF
QDIKKPAEDEWKGKTPDAMKAAMALEKKLNQALLDLHALGS
ARTDPHLCDFLETHFLDEEVKLIKMGDHLTNLHRLGGPEA
GLGEYLFERLTLKHD

B:

Primer Sequence	Function
TAGCGCGGGTGGTAGCGGTGGAGACAGCATGACGACCGCGTCCACCTCGCAGGTG	Ferritin H forward
TAATTCTGACGAATCTGGGAGCTCATGCTTTTCATTATCACTGTCTCCAGGG	Ferritin H reverse
CCCTGGGAGACAGTGATAATGAAAGCATGAGCTCCAGATTCGTCAGAATTA	Ferritin L forward
TTTTTTGAATTCTCATTAGTCGTGCTTGAGAGTGAGCCTTTTCGAA	Ferritin L reverse with EcoR1
GTCTCCACCGCTACCACCCGCGCTACCCGGTTTGATTTCCACCTTGGTCCCTTGCCCG	scFv reverse with Ferritin overlap

Figure 3.

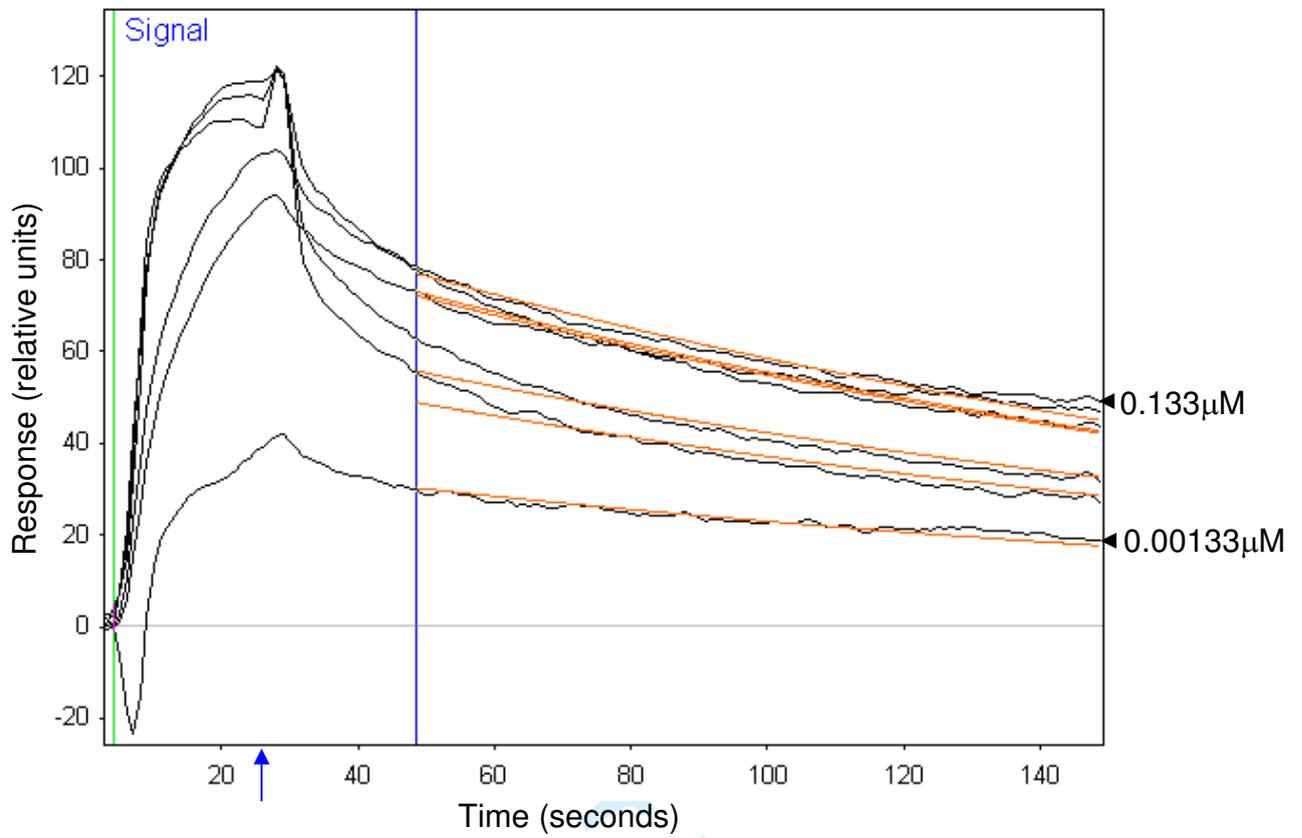


Figure 4.

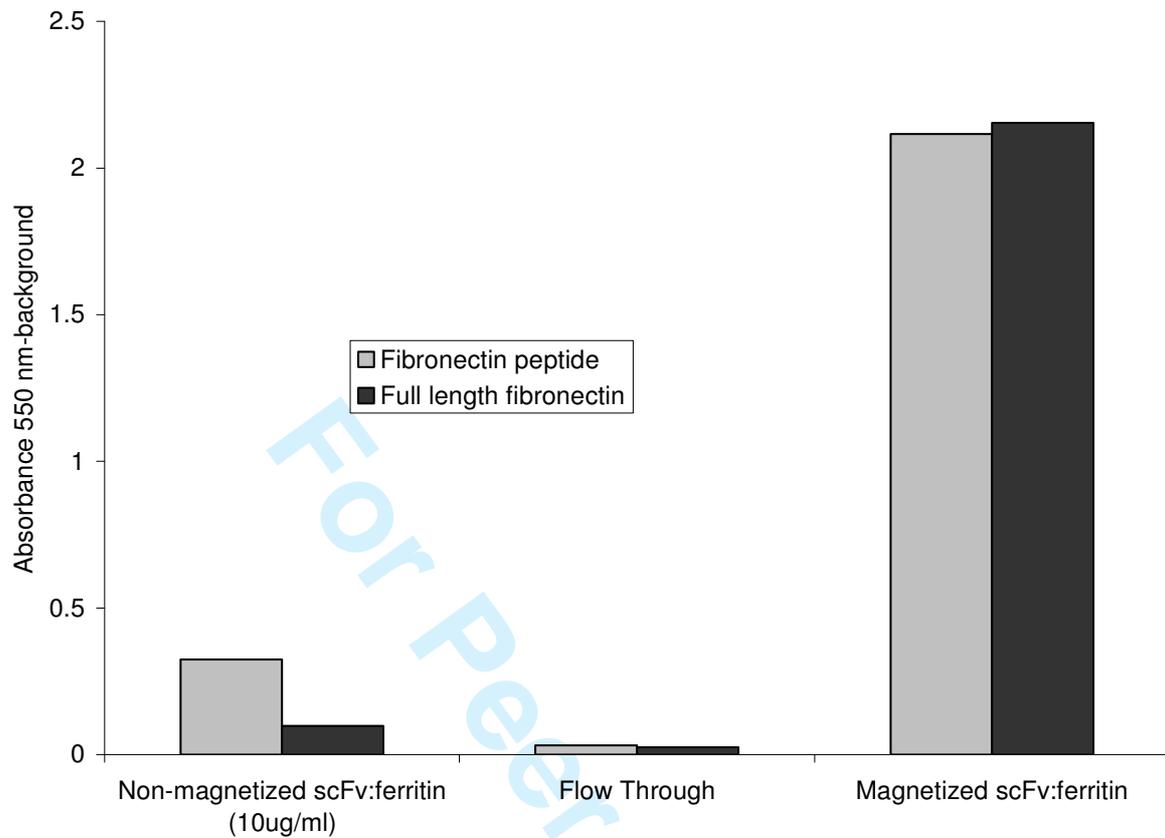
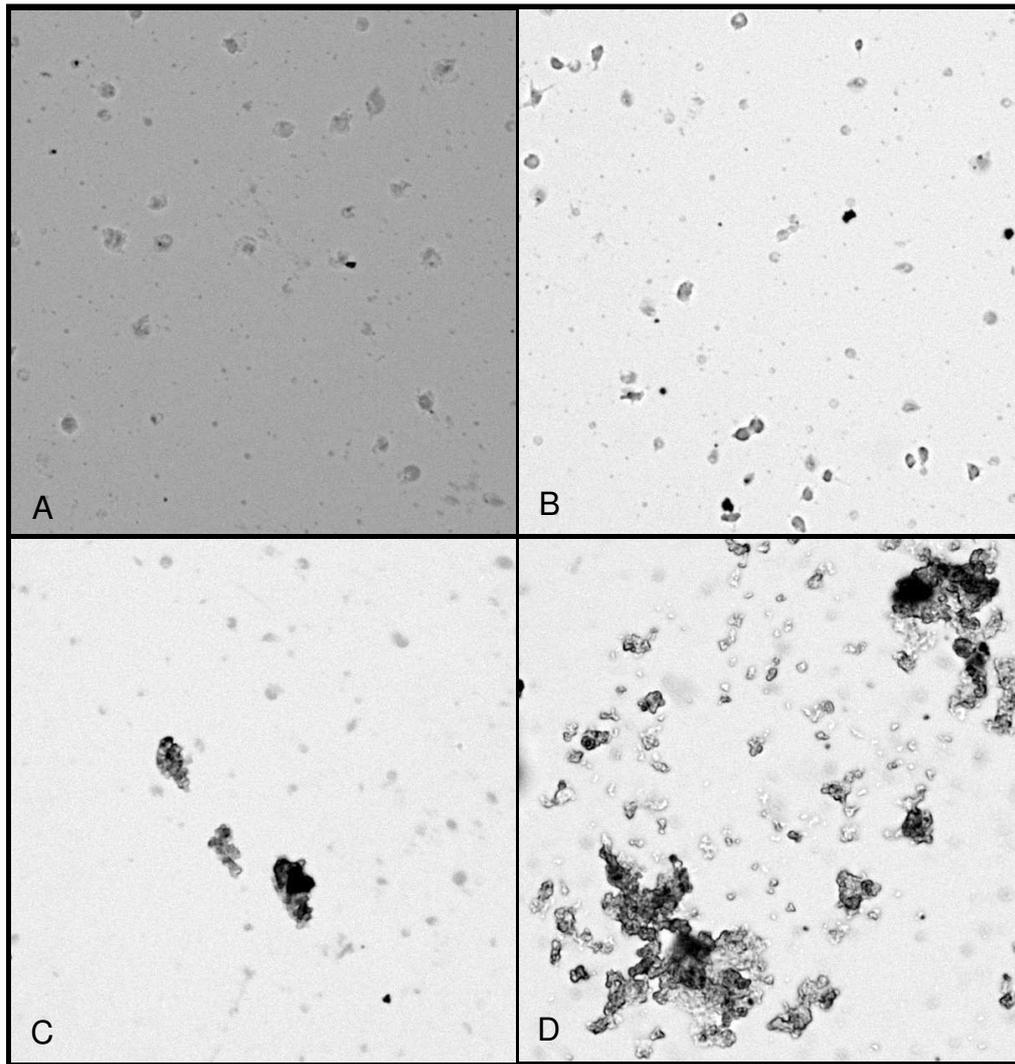
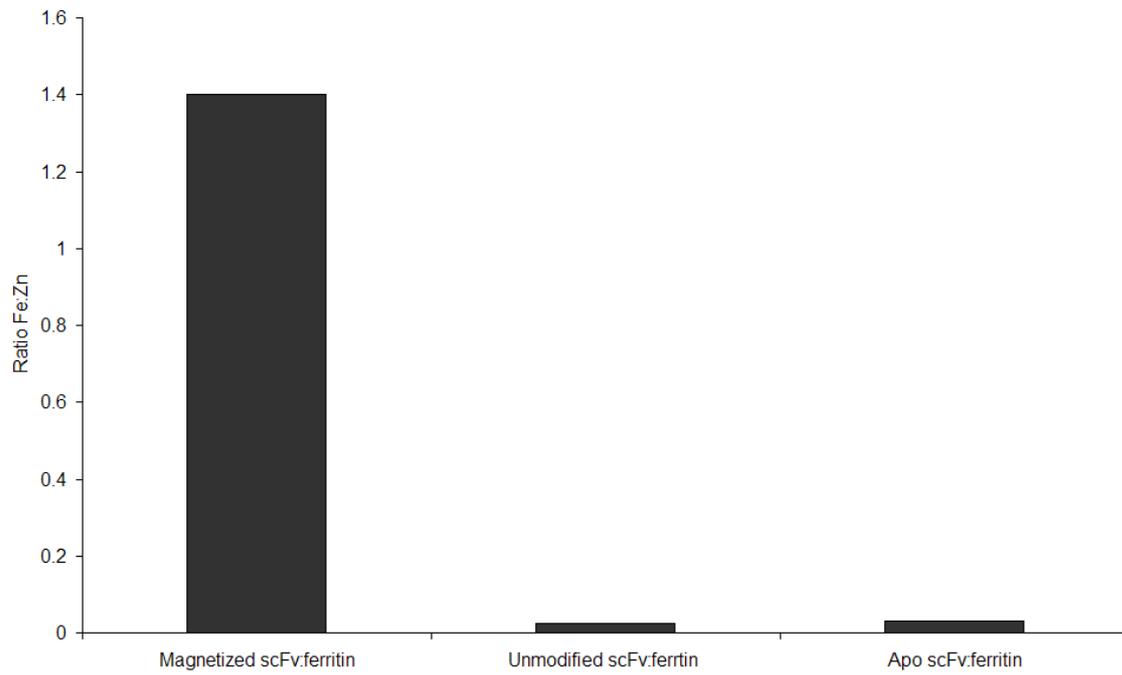


Figure 5.



view

Figure 6.



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