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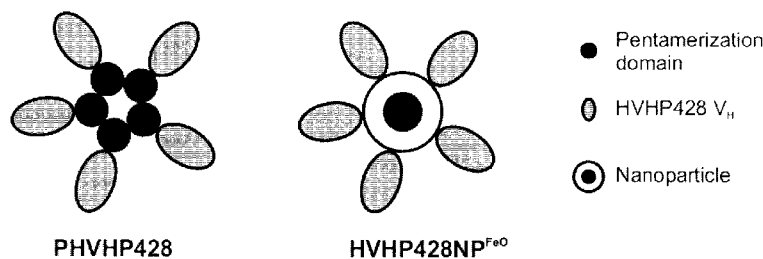


FIG. 2

(57) Abstract: A conjugate comprising a superparamagnetic nanoparticle (SPN) coupled to at least one single-domain antibody (sd Ab) specific to a pathogen or cell of interest. The SPN may comprise an iron oxide core and a silica shell and may be labelled with SERS-active Raman reporter molecule, or a detectable label.

SINGLE DOMAIN ANTIBODY - TARGETED NANOPARTICLE ARCHITECTURES FOR
INCREASED PATHOGEN DETECTION
SPECIFICITY AND SENSITIVITY

5 **Field of the Invention**

The present invention relates to targeted nanoparticle architectures for use in detecting pathogens with high specificity and sensitivity. More specifically, the present invention relates to a pathogen-detecting targeted superparamagnetic nanoparticle (SPN).

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Background of the Invention

Antibodies are highly target-specific. Nanoparticles possess diverse, material-dependent properties that can be exploited in order to label and identify a biomolecule. In combination, antibody-nanoparticle conjugates (nanoconjugates) should be conducive to detection formats characterized by high target specificity, sensitivity and fast detection/identification output. There are examples where such nanoconjugates have been employed in the labeling and detection of cancer cells (Medley et al, 2008) and biomarkers for debilitating diseases such as Alzheimer's (Lin et al, 2006; Georganopoulou et al, 2005).

Nanoconjugates would be of great benefit in the field of bacterial pathogen detection (Gu et al, 2006; Kalele et al, 2006; Ho et al, 2004; Su et al, 2004; Tu et al, 2003; Tu et al, 2002). Some prime examples for detection are those that are biological threat agents or food pathogens such as *Bacillus anthracis*, *Francisella tularensis*, *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Campylobacter jejuni*, *Lysteria monocytogenes*, and *Staphylococcus aureus*. Effective and timely preventive measures against such bacteria require that they be detected in trace amounts, thus calling for a detection system which is highly specific, sensitive and fast.

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Use of antibody nanoconjugates can lead to the effective isolation of a number of pathogen species, there is generally an inherent cross reactivity to traditional IgG

antibodies. This can be somewhat problematic when a single species of bacteria is to be isolated. This was demonstrated by Ho and coworkers where an IgG antibody was shown to interact with at least 3 different species of bacteria (Ho et al, 2004).

- 5 Therefore, there remains a need in the art for a substrate and method that mitigate these limitations of the prior art.

Summary of the Invention

- 10 The present invention relates to targeted nanoparticle architectures for use in detecting pathogens with high specificity and sensitivity. More specifically, the present invention relates to a pathogen-detecting targeted superparamagnetic nanoparticle (SPN). The applicant has determined that the superparamagnetic nanoconjugates described herein are an attractive option as pathogen detection constructs. By virtue of their magnetic property
15 they can magnetically confine/preconcentrate cell samples, leading to increase in detection sensitivity. With a dye doped version employed, the detection sensitivity may be increased further through signal amplification (i.e., every binding event would lead to the “deposition” of thousands of detecting dyes (signals) on the surface of cells) (Zhao et al, 2004).

20

In one embodiment, the basis of pathogen recognition is an antibody-antigen immune or pseudoimmune reaction (Ho et al., 2004). In one embodiment, the targeting moiety is a single domain antibody (sdAb).

- 25 The present invention provides a conjugate comprising a superparamagnetic core comprising iron oxide nanoparticles, coupled to at least one single-domain antibody (sdAb) specific to a pathogen or cell of interest. The sdAb may be specific to protein A on the surface of *Staphylococcus aureus*, in particular the methicillin-resistant varieties (MRSA) and may be used for their detection. In one embodiment, the sdAb may
30 comprise the sequence:

QLQLQESGGGLVQPGGSLRLSCAASGFTFSSYAMSWFRQAPGKGLEWVG
 FIRSKAYGGTTEYAASVKGRFTISRDDSKSIAYLQMNSLRAEDTAMYYCA
 RRAKDGYNPEDIYWGQGTLVTVSS [SEQ ID. NO. 1]

or a substantially identical sequence thereto. In another embodiment, the sdAb may be
 5 sdAb HVHP428.

In another aspect, the present invention comprises a method of detecting a pathogen of
 interest in a mixed culture or sample, by binding the pathogen with a conjugate described
 herein, magnetically concentrating the pathogens bound with the conjugate, and detecting
 10 the pathogens bound with the conjugate. The conjugate may further comprise a Surface-
 enhanced Raman scattering (SERS)-active Raman reporter molecule. In addition, or
 alternatively, the SPNs may further comprise a detectable label, such as a fluorescent dye.

In another aspect, the invention provides the use of conjugates as described herein to
 15 separate and purify a target of interest in a mixture or a sample. The target of interest
 may be cells such as mammalian cells, to be separated from a mixture containing several
 different types of cells.

Brief Description of the Drawings

20 In the drawings, like elements are assigned like reference numerals. The drawings are
 not necessarily to scale, with the emphasis instead placed upon the principles of the
 present invention. Additionally, the embodiments depicted are but few of a number of
 25 possible arrangements utilizing the fundamental concepts of the present invention. The
 drawings are briefly described as follows:

Figure 1 shows different antibody formats. An antibody, for example an IgG, consists of
 two identical heavy chains (hatched and grey) and two identical light chains (black and
 30 white). Antibody domains are shown as ovals. The Fc region of the IgG, which consists
 of four domains, interacts with *S. aureus* protein A as well as protein G and protein M
 present on the surface of other bacteria (Ho et al, 2004; Tashiro et al, 1995; Burova et al,

1999); this is known as the pseudoimmune reaction. The Fv region (white and grey) of the IgG binds the antigen through hypervariable loops/complementarity-determining regions (immune reaction). A scFv (a light chain binding domain plus a heavy chain binding domain covalently joined by a linker) maintains the antigen binding activity of the parent antibody. In many scFvs, the two binding domains can dissociate then re-associate intermolecularly; on the surface of nanoparticles, this would lead to agglutination. V_H , which is over 10-fold smaller than an IgG, also has the ability to bind to antigen; some also have the ability to bind to protein A (pseudoimmune reaction). The smaller V_H s display a lower number of reactive sites and as a result, unlike larger IgG or scFv, lead to insignificant nanoparticle agglutination during conjugation.

Figure 2 is a schematic showing the HVHP428^P pentamer versus the HVHP428SPN^{Fe2O3} superparamagnetic nanoconjugate. Pentameric V_H s are generated recombinantly *in vivo*, e.g., in *E. coli*, by the spontaneous assembly of five identical monomeric units. The assembly is mediated by the specific interaction of the pentamerization domains which are covalently fused to the V_H s at the gene level. In the case of the present nanoconjugates, multivalency is achieved by chemical linking, via amide linkages, of several V_H s to the same nanoparticle. A magnetic core allows for the magnetic pre-concentration of pathogens and the introduction of a dye-doped silica shell would allow for the fluorescent detection of the pre-concentrated pathogen.

Figure 3 shows results of microagglutination assays for HVHP428^P, HVLP335^P and HVHP428SPN^{Fe2O3}. A constant concentration of *S. aureus* cells was used, while the concentrations of pentamers and nanoparticles decrease two-fold from well 1 to well 11 with well 12 having only MES buffer. The concentrations of the pentamers and nanoparticles in wells 1 are 215 $\mu\text{g/mL}$ and 2.5×10^{12} particles/mL, respectively. **Figure 3A** shows results for HVHP428^P and HVLP335^P. In **Figure 3B**, MAC values were 3×10^{13} pentamers/mL and 1.5×10^{11} particles/mL for HVHP428^P and HVHP428SPN^{Fe2O3}, respectively.

Figure 4 shows results of flow cytometry analysis of HVHP428SPN^{Fe2O3}. Nanoparticles were incubated with 0, 0.05, 0.1, 0.2, 0.5 and 1 µg of fluorescently active protein A (protein A R-PE) and assessed directly for binding by flow cytometry. **(Figure 4A)** Binding, indicated as the rightward fluorescence shifts of nanoparticle populations, can be seen with the increase in protein A R-PE concentration. **(Figure 4B)** Binding is shown as the mean fluorescence, extracted from data in **(Figure 4A)**, versus protein A R-PE concentration. Circles, HVHP428SPN^{Fe2O3}; triangles, SPN^{Fe2O3}.

Figure 5 shows results of TEM experiments for HVHP428SPN^{Fe2O3}. Representative TEM images of the nanoparticle-bacteria conjugates where there is a strong interaction between HVHP428SPN^{Fe2O3} and *S. aureus* cells **(Figure 5A)** in contrast to the inefficient labeling of *S. typhimurium* by the same nanoparticles **(Figure 5B)**. Control TEM images were also acquired confirming that SPN^{Fe2O3} does not interact effectively with *S. aureus* cells in the absence of HVHP428 **(Figure 5C)**, suggesting that the sdAb mediates the specific interaction between the nanoparticles and the *S. aureus* cells.

Figure 6 illustrates the cell capture capability of the HVHP428SPN^{Fe2O3} nanoconjugate. **Figure 6A** is a scheme representing the steps involved in capture studies. Cells were incubated with HVHP428SPN^{Fe2O3} for binding. Following binding and magnetic capture of nanoparticles, the supernatant is separated from the captured nanoparticles and titrated to determine the number of non-captured cells. The captured nanoparticles were titrated for determining the number of captured cells. Unconjugated nanoparticle, SPN^{Fe2O3} was used as control. Capture efficiency and specificity were calculated as described in Examples. **Figure 6B** shows the capture efficiency of HVHP428SPN^{Fe2O3} nanoconjugate towards *S. aureus*. The data are an average of 5 trials. The number of cells subjected to capture studies ranged from 180 – 4000. **Figure 6C** shows the capture specificity of HVHP428SPN^{Fe2O3} in terms of the ratio of the number of *S. aureus* to *S. typhimurium* in the captured fraction. A mixture of both bacteria was used for capture studies and selective media were used to enumerate each of the two bacteria in the captured and non-captured fractions. *S. aureus* and *S. typhimurium* data were normalized for the total number of cells. The total number of cells in the HVHP428SPN^{Fe2O3} mixtures was 1170

S. aureus and 525 *S. typhimurium* cells in one trial and 2780 *S. aureus* and 2420 *S. typhimurium* in the second trial. The inset shows the number of cells (average of the two trials) captured with HVHP428SPN^{Fe2O3} (white bars) and SPN^{Fe2O3} (black bars).

5 **Figure 7** shows the cell capture efficiency and specificity of HVHP428SPN^{Fe2O3} nanoconjugate when a single wash step is included after the binding step. **Figure 7A** is a bar graph showing the total number of *S. aureus* cells was 338 in the HVHP428SPN^{Fe2O3} mixture and 278 in the SPN^{Fe2O3} mixture. The wash step removed 6 *S. aureus* from the HVHP428SPN^{Fe2O3} mixture and 24 from the SPN^{Fe2O3} mixture. **Figure 7B** shows the
10 capture specificity by comparing the number of *S. aureus* versus *S. typhimurium* in the mixed capture fraction. The total number of cells in the HVHP428SPN^{Fe2O3} mixture was 80 *S. aureus* and 56 *S. typhimurium*. The wash step removed 2 *S. aureus* and 4 *S. typhimurium*.

15 **Figure 8** shows surface plasmon resonance assessment of the binding activity of V_H and V_L pentamers. Sensorgram overlays showing the binding of HVHP428^P to immobilized protein A at 1, 2, 3, 4, 6, 8 and 10 nM concentrations (**Figure 8A**) and of HVLP335^P to immobilized protein L at 1, 2, 2.5, 3, 3.5, 4 and 4.5 nM concentrations (**Figure 8B**). Association and dissociation rate constants, k_a and k_d , respectively, and equilibrium
20 constants, K_A , for the binding of HVHP428^P and HVLP335^P to proteins A and L, respectively, are shown on the graphs. k_a s were independently calculated from plots of k_{obs} versus concentration. More than one k_d could be calculated due to the heterogeneity in multivalent binding amongst the pentamer population. Therefore, more than one K_A , could be obtained. HVHP428^P and HVLP335^P had minimum K_A s of $5 \times 10^8 \text{ M}^{-1}$ and $5 \times$
25 10^9 M^{-1} , respectively. With slower k_d s, HVHP428^P and HVLP335^P had K_A s as high as $1.1 \times 10^9 \text{ M}^{-1}$ and $1.1 \times 10^{10} \text{ M}^{-1}$, respectively. Thus, HVHP428^P pentamer shows increased apparent affinity towards protein A by at least 1000-fold compared to the monomeric version, HVHP428. RU, resonance units.

30 **Figure 9** shows results of microagglutination assays and TEM experiments assessing the cross-reactivity of HVHP428SPN^{Fe2O3} towards *S. saprophyticus*, *S. pneumoniae* and *S.*

pyogenes. The concentrations of HVHP428SPN^{Fe2O3} (odd rows) and HVHP428SPN^{Fe2O3} (even rows) decrease two-fold from well 1 to well 3 with well 4 having only MES buffer. *S. pyogenes* did not lend itself to the microagglutination assay and therefore its cross reactivity status was assessed by TEM experiments (left panel images). TEM images show lack of interactions between both HVHP428SPN^{Fe2O3} and SPN^{Fe2O3} and *S. pyogenes* cells in contrast to the efficient labeling of *S. aureus* by HVHP428SPN^{Fe2O3} (**Figure 5A**). Though there are nanoparticles present in the TEM image of the *S. pyogenes* bacteria, the nanoparticles are clearly not interacting with the surface of these cells to the extent that they do with *S. aureus*.

Figure 10 shows results of SEM experiments to assess the cross reactivity of HVHP428SPN^{Fe2O3} towards *S. saprophyticus*, *S. pneumoniae* and *S. pyogenes*. Cells and nanoparticles are shown by black and white arrows, respectively. The scale bar corresponds to 1 μ m. 1-SPN = HVHP428SPN^{Fe2O3}; SPN = SPN^{Fe2O3}.

Figure 11 shows the UV-visible absorption spectra of HVHP428SPN^{Fe2O3}. (a) HVHP428SPN^{Fe2O3}; (b) one-day-old HVHP428SPN^{Fe2O3} reacted with tetramethylrhodamine isothiocyanate (TRITC); (c) and one year-old HVHP428SPN^{Fe2O3} reacted with TRITC; (d) magnetically confined one-day-old HVHP428SPN^{Fe2O3} reacted with TRITC; (e) magnetically confined one year-old HVHP428SPN^{Fe2O3} reacted with TRITC; (f) corrected absorption spectra for one-day-old HVHP428SPN^{Fe2O3} reacted with TRITC (i.e., b – a); and (g) (f) corrected absorption spectra for one-year-old HVHP428SPN^{Fe2O3} reacted with TRITC (i.e., c – a). Note that when the nanoparticle absorption is subtracted, there is a clear absorption consistent with tetramethylrhodamine (TMR) (inset). Upon magnetic confinement, the absorption intensity of both the nanoparticle and TMR have decreased (bottom two spectra) suggesting that the TMR is covalently linked to the HVHP428SPN^{Fe2O3} in both cases.

Figure 12 shows the calibration curve for TRITC in MES buffer used to determine the average number of sdAb on each nanoparticle. The calculations are based on the UV-visible absorption data shown in **Figure 11**.

Detailed Description of Preferred Embodiments

The present invention relates to targeted nanoparticle architectures for use in detecting pathogens with high specificity and sensitivity. More specifically, the invention relates to
5 a pathogen-detecting targeted superparamagnetic nanoparticle (SPN).

When describing the present invention, all terms not defined herein have their common art-recognized meanings. To the extent that the following description is of a specific embodiment or a particular use of the invention, it is intended to be illustrative only, and
10 not limiting of the claimed invention. The following description is intended to cover all alternatives, modifications and equivalents that are included in the spirit and scope of the invention, as defined in the appended claims.

The present invention provides a conjugate comprising a superparamagnetic core
15 comprising, in one embodiment, iron oxide nanoparticles, coupled to at least one sdAb specific to a pathogen of interest. The sdAb may be specific to protein A on the surface of *Staphylococcus aureus*, in particular the methicillin-resistant varieties (MRSA) and may be used for their detection.

20 As used herein, the term "nanoparticle" means a particle having at least one dimension which is less than about 200 nm.

By the term "superparamagnetic nanoparticles" (SPN) or "superparamagnetic core", it is meant nanoparticles that exhibit a behavior similar to paramagnetism at temperatures
25 below the Curie or the Néel temperature. This is a small length-scale phenomenon, where the energy required to change the direction of the magnetic moment of the particle is comparable to the ambient thermal energy. Normally, coupling forces in ferromagnetic materials cause the magnetic moments of neighboring atoms to align, resulting in very large internal magnetic fields. This is what distinguishes ferromagnetic materials from
30 paramagnetic materials. At temperatures above the Curie temperature (or the Neel temperature for antiferromagnetic materials), the thermal energy is sufficient to overcome

the coupling forces, causing the atomic magnetic moments to fluctuate randomly. Because there is no longer any magnetic order, the internal magnetic field no longer exists and the material exhibits paramagnetic behavior. If the material is non-homogeneous, one can observe a mixture of ferromagnetic and paramagnetic clusters of atoms at the same temperature, the superparamagnetic stage. Superparamagnetic nanoparticles are described in C.M. Sorensen (2001), the contents of which are incorporated herein by reference, where permitted.

The SPN may comprise any suitable superparamagnetic nanomaterial; for example, and without wishing to be limiting in any manner, the superparamagnetic material may be Fe_2O_3 , Fe_3O_4 , FePt, or nanoparticle clusters containing any and all combinations of these nanoparticles. In a specific, non-limiting example, the SPN are comprised of Fe_2O_3 nanoparticles. The formation of superparamagnetic nanoparticles is well known by those skilled in the art, and need not be further described herein (see for example Hyeon, 2003, which is incorporated herein by reference where permitted).

The size of the SPN may vary based on the type of material comprising the nanoparticles. As would be understood by a person of skill in the art, depending on the material of which the nanoparticles are comprised, the range of the nanoparticle size may vary. For example, and without wishing to be limiting in any manner, the Fe_2O_3 SPN may have a diameter of about 5 to 20 nm; for example, the diameter of the nanoparticle may be about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nm. In a specific, non-limiting example, the diameter of the SPN core may be about 10 nm.

In one embodiment, the SPN may also be encapsulated in a silica shell or coating. Without wishing to be bound by theory, the silica shell may enable attachment of biomolecules and may reduce toxicity. Thus, the SPN may comprise a core + shell architecture familiar to the skilled artisan. Methods for preparing the silica shell are also well-known to those of skill in the art (see for example, Lu et al, 2002; Kell et al, 2008). For example, and without wishing to be limiting in any manner, the thickness of the silica coating may be applied in a controlled manner over the SPN core. The thickness of the

silica coating, once complete, may be about 1 nm and 20 nm, or any value there between; for example, the silica coating may be about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nm thick. As would be understood by one of skill in the art, as the thickness of the silica shell is increased, the magnetic confinement time for the bacteria will require longer times (Kell et al, 2008). In a specific, non-limiting example, the thickness of the silica coating may be about 15 nm.

The conjugate of the present invention comprises SPNs conjugated to one or more than one single-domain antibody (sdAb). By the term “single-domain antibody”, it is meant an antibody fragment comprising a single protein domain. Single domain antibodies may comprise any variable fragment, including V_L , V_H , V_{HH} , V_{NAR} , and may be naturally-occurring or produced by recombinant technologies. For example V_{HS} , V_{LS} , V_{HHS} , V_{NARS} , may be generated by techniques well known in the art (Holt, et al., 2003; Jespers, et al., 2004a; Jespers, et al., 2004b ; Tanha, et al., 2001 ; Tanha, et al., 2002; Tanha, et al., 2006 ; Revets, et al., 2005 ; Holliger, et al., 2005 ; Harmsen, et al., 2007 ; Liu, et al., 2007 ; Dooley, et al., 2003 ; Nuttall, et al., 2001 ; Nuttall, et al., 2000 ; Hoogenboom, 2005; Arbabi-Ghahroudi et al., 2008). In the recombinant DNA technology approach, libraries of sdAbs may be constructed in a variety of ways, “displayed” in a variety of formats such as phage display, yeast display, ribosome display, and subjected to selection to isolate binders to the targets of interest (panning). Examples of libraries include immune libraries derived from llama, shark or human immunized with the target antigen; non-immune/naïve libraries derived from non-immunized llama, shark or human; or synthetic or semi-synthetic libraries such as V_H , V_L , V_{HH} or V_{NAR} libraries.

In one embodiment, the sdAb may comprise a heavy variable domain (V_H) denoted as HVHP428. HVHP428 belongs to a small subset of V_{HS} that can interact with protein A on *S. aureus* cell surfaces and has binding specificity towards *S. aureus* protein A ($K_A = 5.6 \times 10^5 \text{ M}^{-1}$) (To et al, 2005). The sdAb in the conjugate of the present invention may comprise the amino acid sequence:

QLQLQESGGGLVQPGGSLRLSCAASGFTFSSYAMSWFRQAPGKGLEWVG
 FIRSKAYGGTTEYAASVKGRFTISRDDSKSIAYLQMNSLRAEDTAMYYCA
RRAKDGYNSPEDYWGQGTLVTVSS (SEQ ID NO:1),

- 5 or a sequence substantially identical thereto. The hypervariable loops/complementarity-determining regions (CDRs) are underlined.

A sequence which is substantially identical to another sequence may comprise one or more conservative amino acid mutations. It is known in the art that one or more
 10 conservative amino acid mutations to a reference sequence may yield a mutant polypeptide with no substantial change in physiological, chemical, or functional properties compared to the reference sequence; in such a case, the reference and mutant sequences would be considered “substantially identical” polypeptides. Conservative amino acid mutation may include addition, deletion, or substitution of an amino acid; a
 15 conservative amino acid substitution is defined herein as the substitution of an amino acid residue for another amino acid residue with similar chemical properties (e.g. size, charge, or polarity).

In a non-limiting example, a conservative mutation may be an amino acid substitution.
 20 Such a conservative amino acid substitution may substitute a basic, neutral, hydrophobic, or acidic amino acid for another of the same group. By the term “basic amino acid” it is meant hydrophilic amino acids having a side chain pK value of greater than 7, which are typically positively charged at physiological pH. Basic amino acids include histidine (His or H), arginine (Arg or R), and lysine (Lys or K). By the term “neutral amino acid” (also
 25 “polar amino acid”), it is meant hydrophilic amino acids having a side chain that is uncharged at physiological pH, but which has at least one bond in which the pair of electrons shared in common by two atoms is held more closely by one of the atoms. Polar amino acids include serine (Ser or S), threonine (Thr or T), cysteine (Cys or C), tyrosine (Tyr or Y), asparagine (Asn or N), and glutamine (Gln or Q). The term “hydrophobic
 30 amino acid” (also “non-polar amino acid”) is meant to include amino acids exhibiting a hydrophobicity of greater than zero according to the normalized consensus

hydrophobicity scale of Eisenberg (1984). Hydrophobic amino acids include proline (Pro or P), isoleucine (Ile or I), phenylalanine (Phe or F), valine (Val or V), leucine (Leu or L), tryptophan (Trp or W), methionine (Met or M), alanine (Ala or A), and glycine (Gly or G). "Acidic amino acid" refers to hydrophilic amino acids having a side chain pK value of less than 7, which are typically negatively charged at physiological pH. Acidic amino acids include glutamate (Glu or E), and aspartate (Asp or D).

Sequence identity is used to evaluate the similarity of two sequences; it is determined by calculating the percent of residues that are the same when the two sequences are aligned for maximum correspondence between residue positions. Any known method may be used to calculate sequence identity; for example, computer software is available to calculate sequence identity. Without wishing to be limiting, sequence identity can be calculated by software such as NCBI BLAST2 service maintained by the Swiss Institute of Bioinformatics (and as found at <http://ca.expasy.org/tools/blast/>), BLAST-P, Blast-N, or FASTA-N, or any other appropriate software that is known in the art.

The substantially identical sequences of the present invention may be at least 75% identical; in another example, the substantially identical sequences may be at least 70, 75, 80, 85, 90, 95, or 100% identical at the amino acid level to sequences described herein. Importantly, the substantially identical sequences retain the activity and specificity of the reference sequence.

The sdAb is conjugated (also referred to herein as "linked" or "coupled") to the SPN. Conjugation of sdAbs to the SPN may be accomplished using methods well known in the art (see for example Hermanson, 1996). Single domain antibodies have several exposed lysine (primary amine) residues, and thus one method of covalently anchoring the sdAb to the carboxylic acid-modified nanoparticle surface is through bioconjugation chemistry. Suitable coupling reagents include 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) which is often used in combination with N-hydroxysuccinimide (NHS). For example, the sdAb as described above may have, or may be engineered to have, one or more lysine residues opposite or away from its antigen binding site, which is

used in covalent conjugation to the nanoparticle surface. In one embodiment, the number of single domain antibodies conjugated to the surface of the nanoparticle is controllable and controlled.

- 5 Alternatively, the sdAb may be conjugated to the nanoparticle surface through an amino acid with a carboxylic acid (i.e., Glu or Asp) on the sdAb and primary amines on the nanoparticle, or through binding of the sdAb (detecting entity) to a molecule, e.g., a protein already attached to the nanoparticle and has binding activity towards the sdAb. For example, this could be an antibody which binds to the sdAb or to tags (C-Myc tag, His6 tag) on the sdAb such as anti-C-Myc or anti-His6 antibodies, or through binding of the biotinylated sdAb to a biotin binder on the surface of nanoparticles, e.g., streptavidin, neutravidin, avidin, extravidin. The sdAb could also be coupled to the nanoparticle by means of nickel-nitrilotriacetic acid chelation to a His6-tag.
- 10
- 15 In another alternative, single-domain antibodies can also be engineered to have cysteines opposite their antigen binding sites. Conjugation via a maleimide cross-linking reaction allows the directional display of single domain antibodies where all single domain antibodies are optimally positioned to bind to their antigens. Amine-terminated nanoparticle is activated with maleimide in DMF followed by an incubation of cysteine-terminated single domain antibody to achieve covalent binding through the formation of sulfide bond formation.
- 20

The number of sdAb molecules conjugated to the surface of the SPN may vary, based on various factors, such as the size of the nanoparticle. In one embodiment, the conjugate of the present invention may comprise at least 1 to 15 sdAb molecules conjugated to the surface of the SPN; for example, the conjugate may carry at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 sdAb moieties linked to the SPN. In a specific, non-limiting embodiment, the conjugate may comprise about 4 to about 12 sdAb molecules. As a person of skill in the art would recognize, it may be possible to conjugate more sdAb molecules to the surface of the nanoparticle, depending on particle size, sdAb size and

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characteristics, and on immobilization efficiency. It is to be noted that each of the sdAb molecules linked to the nanoparticle may be the same, or may differ from one another.

The sdAb in the conjugate of the present invention may be specific to a pathogen of interest. As used herein, the term “pathogen” means any human pathogen or those of animals or plants, including bacteria, eubacteria, archaeobacteria, eukaryotic microorganisms (e.g., protozoa, fungi, yeasts, and molds), viruses, and biological toxins (e.g., bacterial or fungal toxins or plant lectins).

In one non-limiting example, the sdAb may be specific to protein A on the surface of *Staphylococcus aureus*, in particular the methicillin-resistant varieties (MRSA). *S. aureus* is a common human pathogen (Lowy et al, 1998). On its surface, it has covalently linked protein A (Sjoquist et al, 1972), which is (i) highly characteristic of *S. aureus* species, (ii) present in high abundance and (iii) capable of binding to various classes of antibodies, e.g. IgGs, through interaction with antibody Fc regions (Sjoquist et al, 1972; Cheung et al, 1997; Tashiro et al, 1995; Forsgren, 1970; Huang, 2006). These characteristics make protein A an attractive marker for *S. aureus* detection (Ho et al, 2004; Huang, 2006; Fukuda et al, 2000; Mongodin et al, 2000; Chang et al, 1996). Previously, *S. aureus* detection by a pseudoimmune reaction has been based on the interaction between the Fc region of whole antibodies and protein A (Ho et al, 2004; Mongodin et al, 2000).

However, the present invention does not involve the antibody Fc region. Rather, a single-domain antibody (sdAb) is employed as the recognition component of the nanoconjugate (**Figure 1**). Single-domain antibodies (sdAbs) are antibody fragments that possess one binding site compared to two for typical antibodies and are over 10-fold smaller in size (~15 kDa) (Holliger & Hudson, 2005). Though sdAbs of different types with desired specificity similar to traditional antibodies are easily obtainable by recombinant approaches (Holliger & Hudson, 2005), sdAbs have several properties which make them preferable over more popular antibody formats such as IgGs or scFvs (**Figure 1**) as the recognition component of nanoconjugate. First, they are highly stable against proteases and chemical denaturants; under nonphysiological conditions they regain their activity

following removal from denaturing conditions (Tay et al, 2007; Arbabi-Ghahroudi et al, 2005). This allows for flexibility in terms of choosing optimal conjugation chemistry conditions (Tay et al, 2007), leads to a more active end product and translates into more efficacious detection as nanoconjugates come into contact with bacterial samples which
5 may be destabilizing and/or contain proteases. Second, sdAbs can be conjugated on the surface of nanoparticles with a much higher binding site density (> 5 fold compared to IgGs and 2-fold compared to scFvs) and do not promote nanoparticle aggregation expected with scFvs and IgGs (**Figure 1**), resulting in much more active nanoconjugates. Third, sdAbs can be easily engineered to contain amino acid residues for conjugation in
10 an active orientation or for performing a variety of conjugation chemistries (Shen et al, 2008; Shen et al, 2005).

The superparamagnetic nanoparticles described herein may also comprise a detectable label, such as a fluorescent dye or quantum dot, which in addition to target
15 preconcentration would provide detection signals. The conjugates of the present invention may also comprise one or more than one SERS-active Raman reporter molecule (Tay et al, 2007). For example, suitable labels may include, but are not limited to Rhodamine 6G, 4-nitrobenzenethiol, 2-methoxybenzenethiol, 3-methoxybenzenethiol, 4-methoxybenzenethiol, and 2-naphthalenethiol.

20 It is presently shown that HVHP428 V_H-conjugated SPNs (HVHP428SPN^{Fe₂O₃}), a conjugate in accordance with the present invention, displays unprecedented superior properties. The nanoconjugates of the present invention are highly stable, active and specific detecting agents, capable of capturing a few tens of pathogenic bacteria in a
25 mixed cell population with very high specificity and efficiency. The use of sdAb eliminated the cross-reactivity “side effect” observed previously with a nanoconjugate that was based on the same type of recognition interaction but utilized whole antibody as its recognition moiety (Ho et al, 2004). Without wishing to be bound by theory, these nanoconjugates are believed to provide excellent performance as a result of a
30 combination of multivalency and the unique properties of its sdAb component.

The sdAb-SPN conjugates of the present invention may be used to detect a pathogen of interest in a mixed culture or sample, by binding the pathogen with the sdAb-SPN conjugate described herein, magnetically concentrating the sdAb-SPN conjugate bound to the pathogen, and detecting the sdAb-SPN conjugate bound to the pathogen. In one embodiment, the sdAb-SPN further comprises a SERS-active Raman reporter molecule. In another embodiment, the SPNs further comprise a detectable label, such as a fluorescent dye or quantum dot.

As used herein, the term "sample" means a sample that may contain an analyte of interest. A sample may comprise a body fluid or tissue (for example, urine, blood, plasma, serum, saliva, ocular fluid, spinal fluid, gastrointestinal fluid and the like) from humans or animals; plant tissue, environmental sample (for example, municipal and industrial water, sludge, soil, atmospheric air, ambient air, and the like); food; and beverages. By the term "mixed culture", it is meant a mixture of different types of cells, including the target pathogen. The mixed culture may comprise various types of bacterial cells, or a mixture of different cell types.

In another aspect, the invention comprises the use of sdAb-SPN conjugates to detect, separate and purify a target of interest in a mixture or a sample. In one embodiment, the target of interest may be cells such as mammalian cells, to be separated from a mixture containing several different types of cells. These cells, once targeted by the sdAb-SPN conjugates may be magnetically separated and concentrated. For example, cells bearing a specific marker, or diseased cells, may be separated and purified from a mixture comprising non-marker cells, or non-diseased cells.

As will be apparent to those skilled in the art, various modifications, adaptations and variations of the foregoing specific disclosure can be made without departing from the scope of the invention claimed herein.

The following examples are intended to exemplify embodiments of the invention, and not to limit the claimed invention in any manner.

Example 1 – Growth of Cells

Salmonella typhimurium (ATCC19585), *Staphylococcus aureus* (ATCC12598), *Streptococcus pyogenes* (ATCC12385) and *Staphylococcus saprophyticus* (ATCC15305) were obtained from American Type Culture Collection (**Manassas, VA**). *Streptococcus pneumoniae* 35C was from an in house stock (NRCC 4758). Xylose lysine desoxycholate (XLD), Baird-Parker (BP) agar and chocolate agar plates were purchased from Oxoid (**Nepean, ON, Canada**). Protein A R-PE conjugate (1 mg/mL) was purchased from Innova Biosciences Ltd. (**Babraham, Cambridge, UK**).

- 10 A single *S. aureus* colony from a plate of brain heart infusion (BHI) media (**EMD Chemicals Inc., Darmstadt, Germany**) was used to inoculate 15 mL of BHI media. The bacteria were grown overnight at 37°C and 200 rpm. In the morning, the culture was spun down in a fixed rotor, Sorval RT6000B refrigerated centrifuge at 4,000 rpm for 10 min, the supernatant was removed and the cell pellet was re-suspended in an appropriate buffer. The cells were re-spun, the supernatant was removed and the cell pellet was re-suspended followed by measuring cell density at OD₆₀₀. Serial dilutions of the cells were spread on BHI plates at 37°C for overnight growth. The cell titer was determined in the morning. An OD₆₀₀ of 1.0 corresponded to 1 x 10⁸ cells/mL. *S. typhimurium* was grown as described for *S. aureus* but with nutrient broth media (NB, 5 g peptone and 3 g meat extract in 1 L water, pH 7.0) (Sambrook et al,1989). For *S. typhimurium*, an OD₆₀₀ of 1 corresponded to 3 x 10⁸ cells/mL. *Staphylococcus saprophyticus* was prepared as outlined for *S. aureus*. An OD₆₀₀ of 1 corresponded to 1 x 10⁸ cells/mL. *Streptococcus pyogenes* was streaked onto chocolate agar plates from frozen stock and grown at 37°C overnight. Plate contents were scraped into sterile phosphate-buffered saline (PBS) and adjusted to an OD₆₀₀ of 1 which corresponded to 1 x 10⁸ cells/mL. *Streptococcus pneumoniae* was grown in media (Columbia broth 17.5g, Todd Hewitt broth 15g, 1% glucose in 1 litre, pH 7.0) at 37°C, 5% CO₂, without shaking. The cells were spun down as stated for *S. aureus*. An OD₆₀₀ of 1 corresponded to 1 x 10⁸ cells/mL. Cells were used in microagglutination assays, transmission electron microscopy (TEM) experiments, scanning electron microscopy (SEM) experiments and capture studies.

Example 2 - Synthesis of the carboxylic acid-modified superparamagnetic nanoparticle SPN^{Fe2O3}, and its conjugate derivative HVHP428SPN^{Fe2O3}

SPN^{Fe2O3} nanoparticles were synthesized by encapsulating an iron-containing particle with a silica matrix through the hydrolysis and polymerization of tetraethoxyorthosilane under basic conditions; this process was described by Kell & Simard (2007). Briefly, commercially available ferrofluid (0.4 mL water soluble ferrotec EMG403; **Ferrotec, Bedford, NH**) was diluted to 400 mL with Millipore water. The resulting stock solution was further diluted (12 mL diluted to 45 mL) in Millipore water, and the solution was sonicated for 30 min. Following sonication, the ferrofluid solution was transferred to a 500 mL flask containing 400 mL isopropanol and mechanically stirred. Tetraethoxyorthosilane (0.14 mL) was added to the flask and stirred for ~ 1 min at which time 6 mL of concentrated ammonium hydroxide was added. The nanoparticles were then stirred overnight. This lead to a core-shell nanoparticle architecture, where an iron oxide nanoparticle (~10 nm) was encapsulated in a silica shell with a thickness of ~15 nm.

The surface of these nanoparticles was then modified with 3-aminopropyldiethoxymethylsilane (AP-DEMS; provides an amine functional group) and subsequently with succinic anhydride to yield a carboxylic acid functional group. Briefly, six 45 mL samples of the crude nanoparticle solution described above were transferred to 50 mL Falcon tubes, and 1.0 mL of AP-DEMS was added to each tube in order to modify the surface with a functional amine group. The mixture was shaken for 24 h and centrifuged at 5300 g for 20 min in order to precipitate the amine-modified nanoparticles. The contents of all of the tubes were combined and washed with ethanol (15 mL) and dimethylformamide (DMF, 15 mL) through repeated centrifugations. Following purification, the amine-modified nanoparticles were dispersed in a 1% solution of succinic anhydride in DMF and shaken overnight. The resulting carboxylic acid-modified nanoparticles were washed with fresh DMF several times to ensure all succinic anhydride had been removed. The nanoparticles were then dispersed in MES buffer (30 mM MES, 70 mM NaCl, pH 6.0) and centrifuged and re-dispersed in fresh MES buffer. The conversion efficiency for the reaction between the amine and the succinic anhydride was determined by reacting the resulting nanoparticle with fluorescamine (Kell &

Simard (2007), which did not result in a fluorescence response, suggesting that all of the accessible amine moieties had reacted with the succinic anhydride. The carboxylic acid-modified nanoparticles are hereafter referred to as $\text{SPN}^{\text{Fe}_2\text{O}_3}$.

5 The resulting $\text{SPN}^{\text{Fe}_2\text{O}_3}$ were surface modified with HVHP428 by first activating $\text{SPN}^{\text{Fe}_2\text{O}_3}$ (10 mL, 1×10^{14} nanoparticles) with EDC (5 mg) and NHS (5 mg) to yield the NHS-ester modified $\text{SPN}^{\text{Fe}_2\text{O}_3}$. Following purification to remove any unreacted EDC and NHS, the nanoparticles were mixed with HVHP428 (100 μL of a 1.8 mg/mL solution in PBS) and gently vortexed for at least five hours. It is believed that HVHP428 is
10 covalently bound to the nanoparticle surface through primary amines, of which there are 7. Following modification with HVHP428, the resulting HVHP428 $\text{SPN}^{\text{Fe}_2\text{O}_3}$ particles were purified via repeated centrifugation cycles to remove any unbound HVHP428. Finally, the nanoparticles were redispersed in 10 mL of MES buffer (30 mM MES, 70 mM NaCl, pH 6.0).

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Example 3 – Characterization of HVHP428 $\text{SPN}^{\text{Fe}_2\text{O}_3}$

The number of HVHP428 VH anchored to the surface of the $\text{SPN}^{\text{Fe}_2\text{O}_3}$ was elucidated by reaction of the HVHP428 $\text{SPN}^{\text{Fe}_2\text{O}_3}$ (Example 2) with tetramethylrhodamine isothiocyanate (TRITC). The reaction was carried out in MES buffer with
20 HVHP428 $\text{SPN}^{\text{Fe}_2\text{O}_3}$ (6×10^{12} nanoparticles) and TRITC (50 μg , 1.2×10^{-7} mol) and the resulting TMR-modified HVHP428 $\text{SPN}^{\text{Fe}_2\text{O}_3}$ was purified and analyzed by UV-visible spectroscopy in order to elucidate the number of sdAbs on the surface of the nanoparticle. The UV-visible absorption spectra of freshly prepare, day-old HVHP428 $\text{SPN}^{\text{Fe}_2\text{O}_3}$ and one-year-old HVHP428 $\text{SPN}^{\text{Fe}_2\text{O}_3}$ (6×10^{12} nanoparticles/mL) and the original
25 HVHP428 $\text{SPN}^{\text{Fe}_2\text{O}_3}$ are shown in **Figure 11**. Analysis of the spectra indicates that TMR has reacted with the sdAb on HVHP428 $\text{SPN}^{\text{Fe}_2\text{O}_3}$, where there is an increase in absorption centered at 550 nm in comparison to the unmodified HVHP428 $\text{SPN}^{\text{Fe}_2\text{O}_3}$. When the number of TMR molecules was known, the number of sdAb per nanoparticle could be elucidated by assuming that only one of the six amine groups on HVHP428 is
30 responsible for anchoring it to the surface of the nanoparticle. Construction of a

calibration curve for TRITC (**Figure 12**) in MES-buffered water and the comparison of the absorption intensity for the HVHP428SPN^{Fe2O3} samples indicate the following:

Table 1. Calculations to determine the average number of sdAb on each nanoparticle

	New HVHP428SPN ^{Fe2O3} modified with TMR (6 x 10 ¹² particles)	Year-old HVHP428SPN ^{Fe2O3} modified with TMR (6 x 10 ¹² particles)
Absorption	0.031	0.010
# TMR molecules	3.7 x 10 ¹⁴	1.2 x 10 ¹⁴
# TMR molecules per SPN	61	19.7
# TMR molecules per sdAb	5	5
# sdAb per SNP	~12	~4

Based on the present calculations, there are 12 and 4 sdAbs per HVHP428SPN^{Fe2O3} for one-day-old nanoparticles and nanoparticles stored in MES buffer at pH 6 for one year, respectively. The slow loss of HVHP428 over several months is consistent with the slow hydrolysis of silica surface ligands in aqueous solution.

Example 4 – Comparison of HVHP428SPN^{Fe2O3} to a pentameric version.

As the first step towards developing HVHP428SPN^{Fe2O3}, it was decided to construct a homologous benchmark against which the performance of the nanoconjugate could be compared. A multimerization technology was previously developed to convert, by genetic engineering, low affinity monomeric sdAbs to highly sensitive pentameric detecting agents (Zhang et al, 2004b). Thus, a pentameric version of HVHP428, HVHP428^P, was created (**Figure 2**) and validated by surface plasmon resonance (i.e., Biacore analysis) (**Figure 8**).

Standard cloning techniques were used to construct V_H and V_L pentamers, HVHP428^P and HVLP335^P, respectively (Zhang et al, 2004). Pentamers were expressed, purified and quantified as described (Zhang et al, 2004; Pace et al, 1995). The binding kinetics for the

interaction of HVHP428^P with protein A and HVLP335^P with protein L were determined from surface plasmon resonance (SPR) data collected with BIACORE 3000 biosensor system (**GE Healthcare, Baie d'Urfé, QC, Canada**). For the HVHP428^P analysis, 520 resonance units (RUs) of protein A or a Fab reference were immobilized on research
5 grade CM5 sensor chips (**GE Healthcare**). For the HVLP335^P analysis, 680 RUs of protein L or 870 RUs of the Fab reference were immobilized. Immobilizations were carried out at protein concentrations of 25 µg/mL (protein A) or 50 µg/mL (protein L and Fab) in 10 mM sodium acetate buffer pH 4.5, using the amine coupling kit provided by the manufacturer. In all instances, analyses were carried out at 25°C in 10 mM HEPES,
10 pH 7.4, containing 150 mM NaCl, 3 mM EDTA and 0.005% P20 at a flow rate of 20 µL/min, and surfaces were regenerated by washing with 50 mM HCl for 3 s. Data were evaluated using BIAevaluation 4.1 software (**GE Healthcare**). Association rate constants, k_{as} , were independently calculated from plots of K_{obs} vs concentration. More than one dissociation rate constant, k_d , could be calculated due to the heterogeneity in
15 multivalent binding among the pentamer population. Therefore, more than one affinity (K_A) could be obtained (see **Figure 8**).

The HVHP428 sdAb has an affinity (K_A) of $5.6 \times 10^5 \text{ M}^{-1}$ with protein A (To et al, 2005). The apparent K_A , determined by SPR experiments, is increased by at least 1000-fold
20 following the generation of the pentameric version HVHP428^P (**Figure 8**). The resulting HVHP428SPN^{Fe2O3} of Example 2 was also expected to improve the apparent K_A between the SPN-bound HVHP428 and protein A. Elucidation of the apparent K_A for HVHP428SPN^{Fe2O3} was done by microagglutination assay (Example 6).

25 Example 5 - Flow Cytometry

To verify that the interaction between the sdAb-modified nanoparticles and *S. aureus* is indeed mediated through protein A and the HVHP428SPN^{Fe2O3} particles, the particles were incubated with fluorophore-conjugated protein A (protein A R-PE) and analyzed in a series of flow cytometry experiments.

Samples of 100 μL HVHP428SPN^{Fe2O3} or SPN^{Fe2O3} (10^{12} particles) were blocked in PBS/0.1% (v/v) Tween-20 overnight at 4°C. To these, 1 μL aliquots of various concentrations of fluorescently active protein A (protein A R-PE conjugate) were added and the reaction volumes were increased by adding 200 μL of PBS/0.75% Tween-20. The samples were incubated for 1 h at 4°C and were directly used for flow cytometry analysis with a FACSCanto Analyzer (**BD Biosciences, Mississauga, ON, Canada**). The R-PE fluorescence of 100,000 events per sample was analyzed and data were generated using *BD FACSDiva* software (**BD Biosciences**).

As highlighted in **Figure 4**, there is a gradual shift in the population of fluorescently active nanoconjugate for HVHP428SPN^{Fe2O3} as a function of protein A R-PE concentration, whereas the nanoparticle without the HVHP428 V_H on its surface (SPN^{Fe2O3}) does not show any binding activity (i.e. there is no shift in the fluorescence, suggesting the protein A does not interact with the nanoparticles). This strongly suggests that the HVHP428 interacts effectively with protein A.

Example 6 – Microagglutination Experiments

The ability of HVHP428-SPN^{Fe2O3} to effectively interact with protein A on the surface of *S. aureus* was next investigated by means of series of microagglutination experiments.

These experiments typically involve incubating HVHP428SPN^{Fe2O3} with *S. aureus* cells in a microtiter plate.

To test its activity towards *S. aureus*, a cell microagglutination assay was performed. Cell microagglutinations were performed essentially as described (Saito et al, 1995).

Two fold dilutions of pentamers (HVHP428^P) or nanoparticles (HVHP428SPN^{Fe2O3}) were performed in MES buffer from wells 1 to 11 of a microtiter plate. Well 12 had only buffer, and the total volume in each well was 50 μL . Subsequently, 1 OD₆₀₀ unit of cells (see *Growth of cells*) in 50 μL buffer was added to all wells and the plate was incubated overnight at 4°C. In the morning, photos of the plates were taken for further analysis.

Minimum agglutination concentration (MAC), the minimum concentration of

nanoparticles or pentamers that promoted bacterial agglutination, was used to express the agglutination activity of nanoparticles and pentamers.

If functional, HVHP428^P would cross-link and agglutinate cells due to its multivalency.

5 In the absence of agglutination the cells settle out over time and form a dot at the center of the well. If there is agglutination the cells settle out as a sheet. Microagglutination was performed by incubating a constant number of bacterial cells with two-fold dilutions of HVHP428^P (**Figure 3A**), as described above.

10 The pentamer is functional as it agglutinates *S. aureus*, up to well 6. Beyond well 6 the concentration of the pentamer is too low for cross-linking, hence no agglutination (cells appear sediments appear as round dots). HVHP428^P exhibits a MAC value of 3×10^{13} pentamers/mL. A control functional active V_L pentamer, HVLP325^P (**Figure 8**) does not show any agglutination, demonstrating that the activity of the HVHP428^P is due to its V_H
15 binding component and not its pentamerization domains (**Figure 2**).

As highlighted in **Figure 3B**, HVHP428SPN^{Fe2O3} also effectively interacts with *S. aureus* cells (evidenced by the diffuse appearance of cell sediments in wells 1-6) and exhibits a MAC value of 1.5×10^{11} particles/mL, 200-fold better (more strongly) than HVHP428^P
20 pentamer. Importantly, HVHP428SPN^{Fe2O3} is specific to *S. aureus* exclusively, as there is no agglutination (cell dots) in the presence of the other species of bacteria. To verify that the interaction was indeed mediated by HVHP428 anchored to the nanoparticle, SPN^{Fe2O3} was also tested for agglutination, and no cell agglutination occurs at any concentration studied for SPN^{Fe2O3}. The long-term stability of HVHP428SPN^{Fe2O3} is
25 impressive; the MAC values do not decrease even following a year of storage in a pH 6 MES buffer at 4°C.

Example 6 – Transmission Electron Microscopy (TEM) experiments

The binding (or lack of interaction) of both HVHP428SPN^{Fe2O3} and SPN^{Fe2O3} to *S. aureus*
30 cells could also be qualitatively assessed by means of transmission electron microscopy (TEM) analysis. In general, we found that the most effective TEM images of the

HVHP428SPN^{Fe2O3} or SPN^{Fe2O3} interacting with the variety of bacteria could be obtained by extracting ~1 μ L of the solution in the microagglutination plates described above. That is, where the nanoparticles agglutinate effectively, 1 μ L of the solution was extracted and expelled directly onto the TEM grid, dried and analysed via TEM. In the case where poor or no microagglutination is observed, the 1 μ L of solution is extracted from the precipitate that has settled to the bottom of the microtiter plate. Here, because both HVHP428SPN^{Fe2O3} and SPN^{Fe2O3} are colloiddally stable, there are significantly less nanoparticles interacting with the bacteria than is the case where agglutination is effective.

The TEM images of the nanoparticle-cell conjugates were acquired using a Philips CM20 FEG microscope operating at 200 keV.

Examples of these TEM binding experiments are provided in **Figure 5**. From these images, two factors are clear. Firstly, HVHP428SPN^{Fe2O3} (**Figure 5A**) interacts much more effectively over the entire surface of the *S. aureus* cells as opposed to SPN^{Fe2O3} (**Figure 5B**).

Secondly, in agreement with the microagglutination investigation highlighted above, the TEM analysis suggests that there is very little interaction between HVHP428SPN^{Fe2O3} and the *S. typhimurium* cells (**Figure 5C**). Also as expected, there was essentially no interaction between SPN^{Fe2O3} and *S. typhimurium* (data not shown).

Example 8 - Antibody Specificity

A previously-described superparamagnetic nanoconjugate employed a traditional whole antibody (IgG), where the interaction between the nanoconjugate and *S. aureus* was mediated through the Fc-protein A pseudoimmune reaction (**Figure 1**) (Ho et al, 2004). It was reported that these whole antibodies showed cross-reactivity with several other bacteria species, including *Staphylococcus saprophyticus* and *Streptococcus pyogenes* (group A *Streptococcus*) (Ho et al, 2004). Such constructs also cross-react with group C and G *Streptococci* through interaction with bacterial protein G (Tashiro et al, 1995).

The microagglutination and TEM experiments were extended to *S. saprophyticus*, *S. pyogenes* and *Streptococcus pneumoniae* (a protein G-containing *Streptococcus*) and we found that the nanoconjugates of the present invention did not cross-react with any of these cells. There is very little evidence for interaction between both HVHP428SPN^{Fe2O3} and SPN^{Fe2O3} nanoparticles and *S. saprophyticus*, *S. pneumoniae* or *S. pyogenes* cells (Figure 9).

Example 9 - Magnetic Confinement Capture Studies

The examples above demonstrate excellent selectivity, therefore, we continued to elucidate how effectively and selectively the nanoparticles could magnetically confine very low concentrations of *S. aureus* cells in competition with other bacterial cells. In these experiments (Figure 6A), HVHP428SPN^{Fe2O3} particles were incubated with *S. aureus* (and *S. typhimurium*) cells for binding/labeling and were subsequently confined into a very small volume by means of a permanent rare-earth magnet. The number of *S. aureus* cells that can be confined from a given sample was determined by cell titration and used to determine capture efficiency and specificity.

HVHP428SPN^{Fe2O3} and SPN^{Fe2O3} were first blocked with 0.1% (v/v) Tween 20 in MES buffer overnight at 4°C. One hundred microliters of the blocked nanoparticles (10¹³ particles/mL) were mixed with 1 µL of cells in MES buffer. The mixture was incubated at room temperature for 2 h with gentle rocking followed by a 1 h-long capture on a magnet. Keeping the sample on the magnet stand, the supernatant was removed and plated on BHI media at various dilutions to determine the number of non-captured cells. The nanoparticles were separated from the magnet, resuspended in 100 µL of MES buffer and used to determine the number of captured cells. Capture efficiency was calculated as follows:

Capture efficiency = (no. of captured cells/ [no. of captured cells + no. of non-captured cells]) x 100

In specificity capture studies, 1 µL of each of *S. aureus* and *S. typhimurium* cells was added to 99 µL of overnight blocked conjugated or unconjugated nanoparticles (10¹³

particles/mL) in MES buffer/1% Tween 20. Incubation, nanoparticle capture and fractionation of mixtures into captured and non-captured cells were performed as above. Cell titration was performed as above except that the fractionated cells were plated on both XLD (selective for *S. typhimurium*) and BP (selective for *S. aureus*) media. Capture
 5 specificity was calculated based on the relative number of *S. aureus* and *S. typhimurium* in the captured fraction, after normalizing for the total number of cells:

Capture specificity = no. of *S. aureus* in the captured fraction/no. of *S. typhimurium* in the captured fraction

Alternatively, capture specificity was determined by direct comparison of the number of
 10 captured *S. aureus* versus the number of captured *S. typhimurium* in the mixed cell captured fraction. In some instances a single wash step between the binding step and the fractionation step was included. The wash step comprised of (i) resuspending the nanoparticles in 100 μ L MES buffer/1% Tween 20, (ii) capturing the nanoparticles for 30 min, (iii) removing the wash solution, which was subsequently titrated. Control
 15 experiments in which cells (*S. aureus* alone, *S. typhimurium* alone or a mixture of both cells) were incubated with the MES buffer had the same total titer as the nanoparticle-treated cells.

Initially, a titration of HVHP428SPN^{Fe2O3} against *S. aureus* cells (10^4 cells) was
 20 performed. Interestingly, we require at least 1×10^{12} HVHP428SPN^{Fe2O3} particles per 10^4 cells in order to label the *S. aureus* cells with sufficient superparamagnetic nanoparticles to facilitate efficient confinement of the cells. No capture was observed at nanoparticle concentrations of 10^3 - 10^{11} particles/mL (capture efficiency: <1%). Non-specific capture could be completely suppressed by coating nanoparticles overnight with
 25 0.1% Tween 20 and performing the binding in the presence of 1% Tween 20. As can be seen in **Figure 6B**, the HVHP428SPN^{Fe2O3} capture efficiency is over 90%, with as few as 180 cells. The capture is specific as evidenced by the poor *S. aureus* capture efficiency exhibited by SPN^{Fe2O3} (~5%).

To determine if the magnetic confinement is specific to *S. aureus*, specificity capture studies involving competition assays containing both *S. aureus* and *S. typhimurium* cells were performed. Mixtures of both bacteria were incubated with nanoparticles for binding/labeling and the captured cells were enumerated by applying bacterial samples
 5 onto selective plates (BP for *S. aureus*, XLD for *S. typhimurium*). The ratio of the *S. aureus* to *S. typhimurium* in the captured fraction was used to determine capture specificity. As can be seen in **Figure 6C**, HVHP428SPN^{Fe2O3} discriminately captures *S. aureus* over *S. typhimurium* with a ratio of 13:1. The number of *S. typhimurium* cells captured by HVHP428SPN^{Fe2O3} is at the background level and is essentially the same as
 10 the numbers captured by SPN^{Fe2O3} in the case of both bacteria (**Figure 6C, inset**).

The selectivity could be further improved simply by including a wash step immediately following the binding step. As highlighted in **Figure 7A**, the capture efficiency was as high as 98% for HVHP428SPN^{Fe2O3} versus only 0.8% for the SPN^{Fe2O3}. (The total
 15 number of *S. aureus* cells was 338 in the HVHP428SPN^{Fe2O3} mixture and 278 in the SPN^{Fe2O3} mixture). Without the wash step HVHP428SPN^{Fe2O3} capture efficiency would have remained at exactly the same level, but that of SPN^{Fe2O3} would have been as high as over 9% (see **Figure 7** legend). This demonstrates that there is very little nonspecific absorption contributing to the interaction between HVHP428SPN^{Fe2O3} and the *S. aureus*
 20 cells. In the case of specificity capture studies, we used a mixture of cells with as few as 80 *S. aureus* cells and 56 *S. typhimurium* cells. The capture specificity was adequate (80 *S. aureus*/4 *S. typhimurium*) immediately following incubation and magnetic confinement and extremely high following the washing step after confinement (78 *S. aureus* and no *S. typhimurium*). Thus, without the wash step the capture specificity ratio would have been
 25 significantly lower.

Example 10 – Capture / scanning electron microscopy (SEM) experiments

We confirmed the specificity of HVHP428SPN^{Fe2O3} through capture/SEM experiments with *S. saprophyticus*, *S. pyogenes*, and *S. pneumoniae* (a protein G-containing
 30 *Streptococcus* species of bacteria).

- Cell were grown as described in Example 1, centrifuged in a benchtop centrifuge (800 g, 5 min), resuspended in ½ MES buffer and pelleted again. Cells were resuspended in 10^{12} HVHP428SPN^{Fe₂O₃} in MES buffer/0.1% Tween 20 (v/v) and incubated for binding for 30 min. The solutions were magnetically confined for 3 min and the supernatant removed -
- 5 leaving the last 20 µL behind to ensure the confined materials are not disturbed and lost. The captured materials were resuspended in 200 µL MES buffer. This step was repeated but the captured materials were resuspended in 200 µL sterile double-distilled H₂O (ddH₂O). The magnetic confinement was performed once more and the captured materials were resuspended in 50 µL ddH₂O. One microliter suspensions were used for
- 10 SEM imaging. SEM was performed on cells plated on Si substrate with a Hitachi S-4700 field-emission scanning electron microscope. Samples were imaged with an acceleration voltage of 3 KV and at a working distance of 6 mm. Identical experiments were carried out with SPN^{Fe₂O₃}.
- 15 Mixtures of nanoparticles (10^{12}) and cells (10^8) were incubated for binding and were subsequently subjected to magnetic confinement, as just described. While in the case of HVHP428SPN^{Fe₂O₃}/*S. aureus*, the solution became clear in a couple of minutes following the magnetic confinement with cells clumped against the vessel wall, indicating a very strong binding interaction between HVHP428SPN^{Fe₂O₃} and *S. aureus*, in the case of
- 20 SPN^{Fe₂O₃}/*S. aureus*, the solution stayed cloudy over the course of the confinement and there was no visible captured cells. With *S. pyogenes* and *S. pneumoniae*, both HVHP428SPN^{Fe₂O₃} and SPN^{Fe₂O₃} hardly gave any captured cells and by the end of the two washes (cycles of resuspension and confinement) there was no visible pack of confined cells. Only in the case of *S. saprophyticus*, some capture was observed, albeit
- 25 with a significant amount of cells still in the supernatant fraction. However, the capture was the same for both HVHP428SPN^{Fe₂O₃} and SPN^{Fe₂O₃}, indicating some non-specific interactions between SPN^{Fe₂O₃} and *S. saprophyticus*. An aliquot of the confined materials following the last wash was used for SEM imaging.
- 30 Figure 10 summarizes results of the capture studies in a series of SEM images. The top panels of Figure 10 depict the interaction of cells with HVHP428SPN^{Fe₂O₃} (labeled 1-

SPN) while bottom panels are the control experiments performed with SPN^{Fe2O3} (labeled SPN). In the *S. aureus* interaction with HVHP428SPN^{Fe2O3} capture study, SEM images showed each of the captured cells (dark sphere, ~1 μ m) were flanked with HVHP428SPN^{Fe2O3} with most cells partially covered by the NP aggregates, which indicated positive binding response between cells and HVHP428SPN^{Fe2O3}. In contrast, in the control experiment (*S. aureus* vs. SPN^{Fe2O3}), very large aggregates of SPN^{Fe2O3} were observed with a few cells embedded in the SPN^{Fe2O3} aggregates. Unlike the *S. aureus* vs. HVHP428SPN^{Fe2O3} interaction, the captured cells in the control experiment had no SPN^{Fe2O3} on their surface nor were they flanked by the nanoparticles. This indicates a lack of interaction between the control nanoparticles and *S. aureus*. It is possible that the few cells trapped in the control experiment were from the remaining 20 μ L of the solution left after the capture step rather than been magnetically confined. SEM images of the interaction between HVHP428SPN^{Fe2O3} with *S. pyogenes* and *S. pneumoniae* showed very similar patterns. Both images show clusters of cells without any nanoparticles binding to their surfaces nor flanked in between the cells which suggests the non-interactive nature of the HVHP428SPN^{Fe2O3} to both *S. pyogenes* and *S. pneumoniae*. Control experiment of both species exposed to SPN^{Fe2O3} also showed similar non-interaction pattern. Despite the observation of very large aggregates of SPN^{Fe2O3} in the images of the control study, there were no nanoparticles binding to the surface of the cells as observed in the case of *S. aureus* vs. HVHP428SPN^{Fe2O3} image. Since there is no binding seen in the images as well as during the earlier confinement step in the case of both *S. pyogenes* and *S. pneumoniae*, it is very likely that the cells seen in the images are carry over from the non-binding fraction (see above). However, some interaction between both HVHP428SPN^{Fe2O3} and SPN^{Fe2O3} with *S. saprophyticus* was suggested by Figure 10. In both *S. saprophyticus* vs. HVHP428SPN^{Fe2O3} and *S. saprophyticus* vs. SPN^{Fe2O3} images, cells are flanked and partially covered by the nanoparticles indicating a positive interaction between the nanoparticles and cell. Because this is observed in both experiments, these interactions are likely non-specific, a conclusion that is also supported by the results of the capture step above. Such non-specific interaction may be further reduced by passivating the nanoparticles surfaces with agents that suppress non-specific interactions such as serum albumins. The interaction between HVHP428SPN^{Fe2O3} and *S.*

saprophyticus may be distinguished from the interaction between HVHP428SPN^{Fe2O3} and *S. aureus* in a real detection situation simply by looking at all of these interactions together.

5 References

The following references are incorporated herein by reference (where permitted) as if reproduced in their entirety. All references are indicative of the level of skill of those skilled in the art to which this invention pertains.

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We claim:

1. A conjugate comprising a superparamagnetic nanoparticle (SPN) coupled to at least one sdAb specific to a pathogen or cell of interest.

5 2. The conjugate of claim 1, wherein the SPN comprises an iron oxide core and a silica shell.

3. The conjugate of claim 1 or 2, wherein the sdAb is specific to protein A on the surface of *Staphylococcus aureus*.

10

4. The conjugate of any one of claims 1 to 3, wherein the sdAb comprises the amino acid sequences;

GFTFSSYAMS, FIRSKAYGGTTEYAASVKG, and RAKDGYNSPEDY

or substantially identical sequences thereto.

15

5. The conjugate of claim 4 wherein the sdAb comprises SEQ ID NO. 1 or a substantially identical sequence thereto.

6. The conjugate of any one of claims 1 to 5, wherein the sdAb is HVHP428.

20

7. The conjugate of any one of claims 1 to 6, wherein the nanoparticle further comprises a SERS-active Raman reporter molecule, or a detectable label.

8. The conjugate of claim 7 wherein the nanoparticle comprises a detectable label comprising a fluorescent label.

25

9. A method of detecting a pathogen of interest in a mixed culture or sample, comprising the steps of:

a) binding the pathogen with a conjugate of any one of claims 1 to 8, wherein the sdAb is specific to the pathogen of interest;

30

b) magnetically concentrating the pathogens bound with the conjugate; and

c) detecting the pathogens bound with the conjugate.

10. A use of a conjugate of any one of claims 1 to 8 wherein the sdAb is specific to the pathogen of interest, for separating and purifying a pathogen of interest in a mixed culture
5 or a sample.

11. A use of a conjugate of any one of claims 1 to 8 for separating mammalian cells from a mixture containing several different types of cells, wherein the sdAb is specific to the mammalian cells of interest.

10

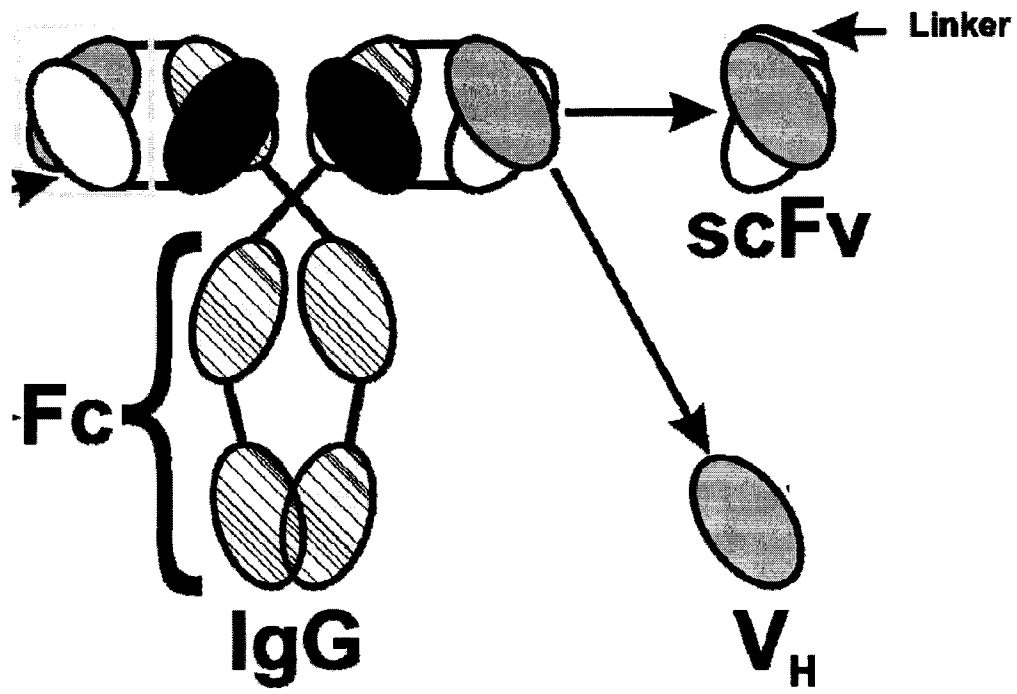


FIG. 1

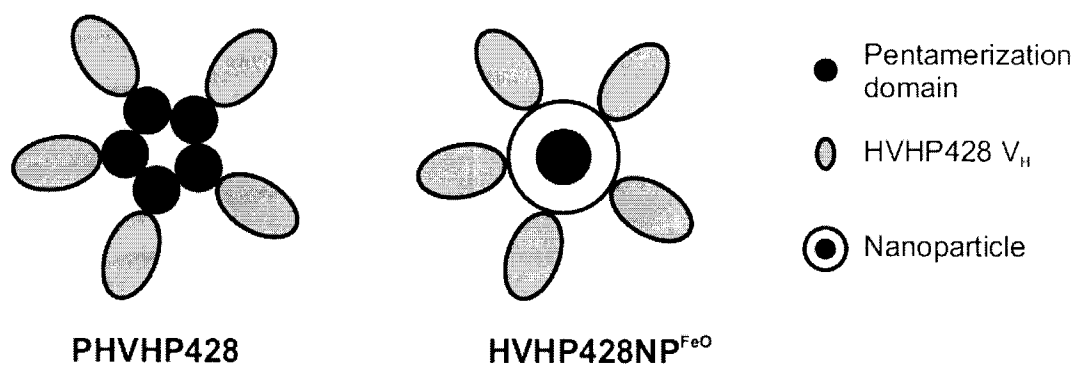


FIG. 2

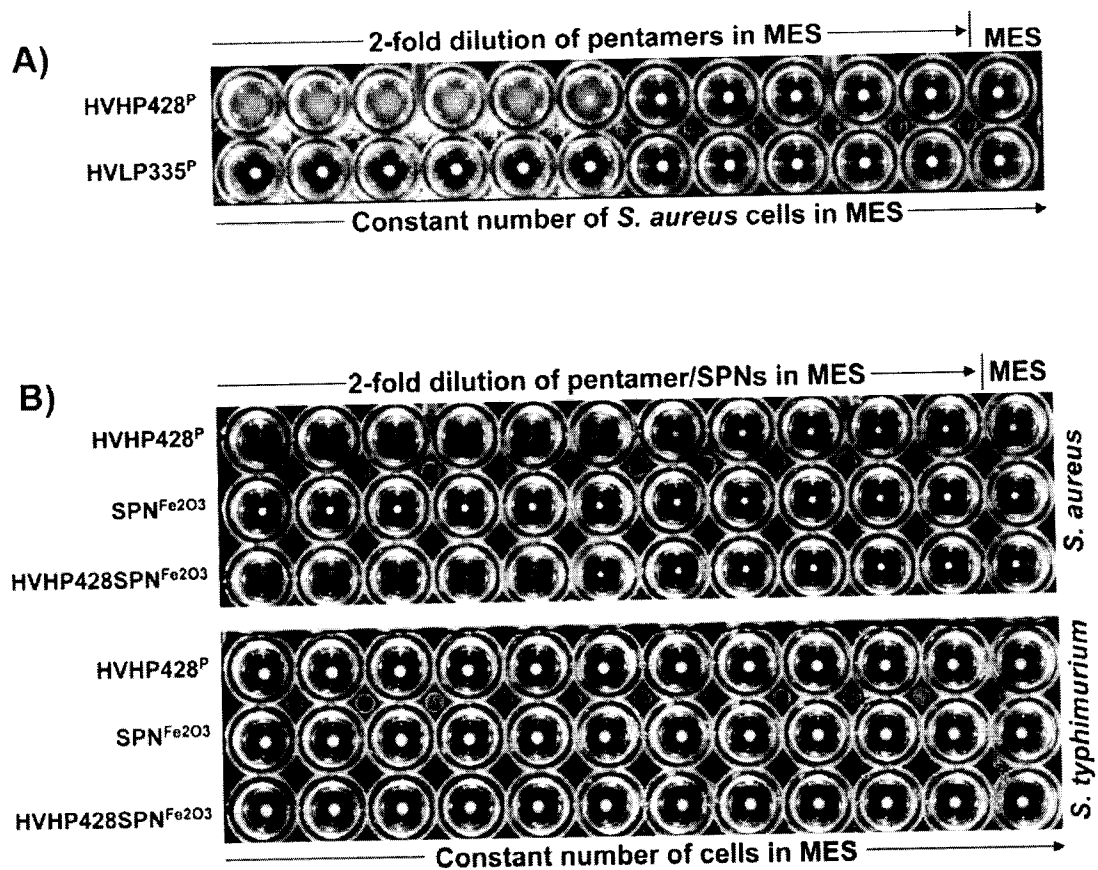


FIG. 3

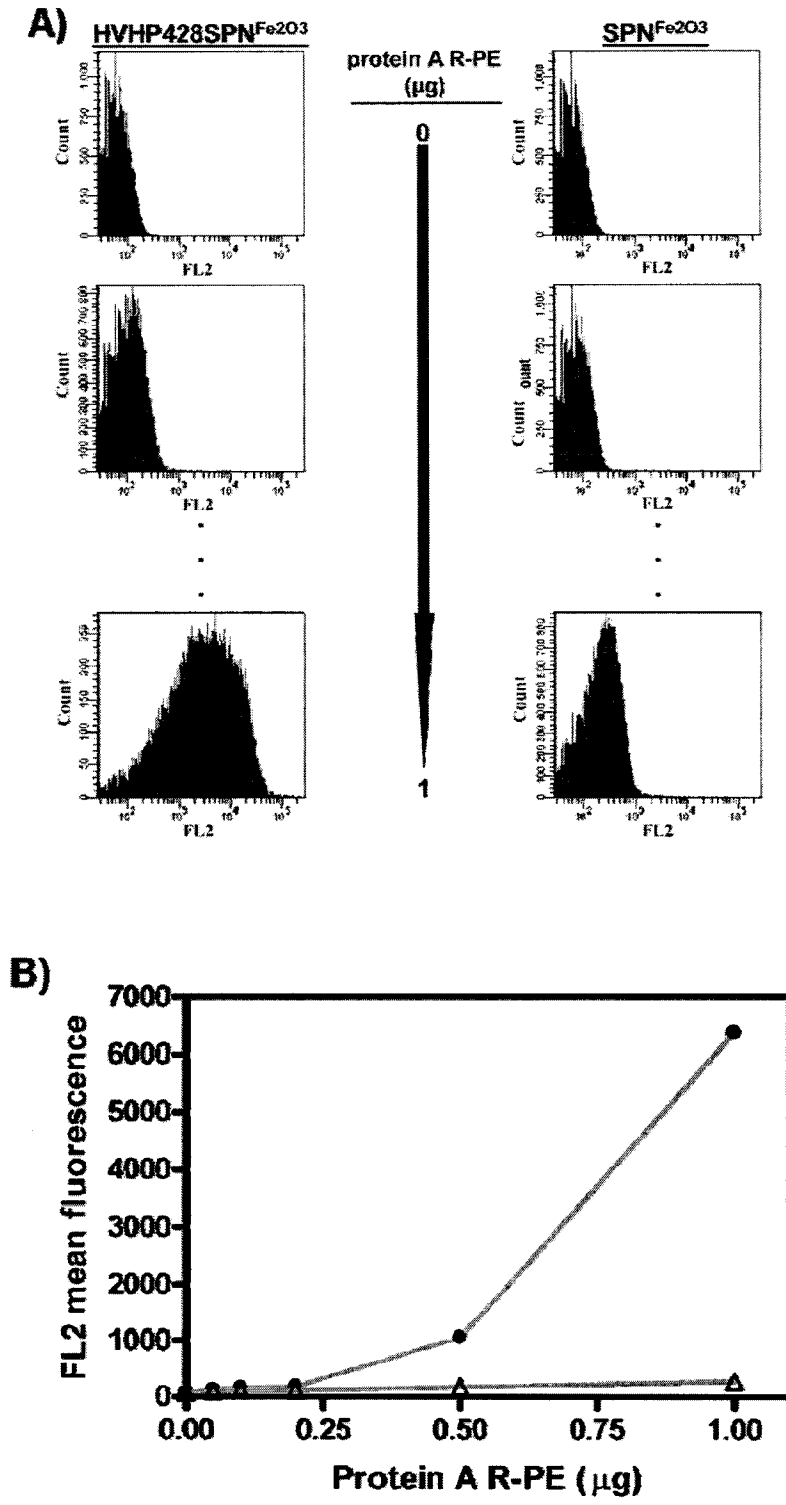


FIG. 4

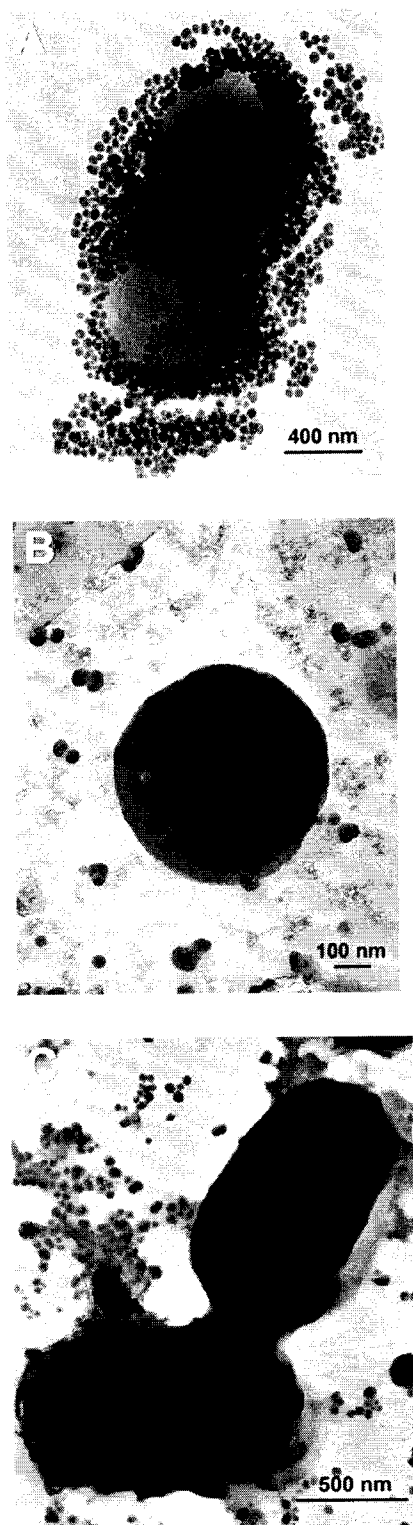


FIG. 5

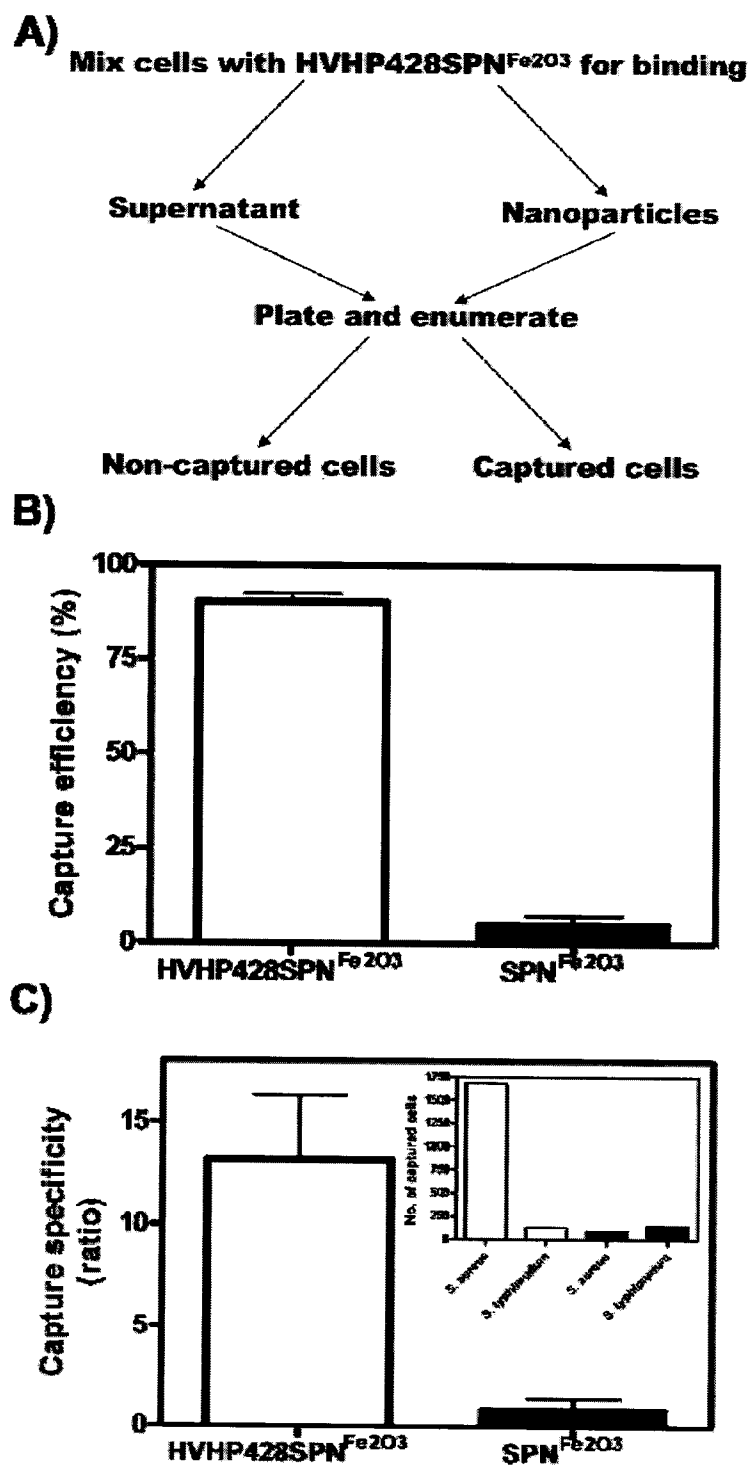


FIG. 6

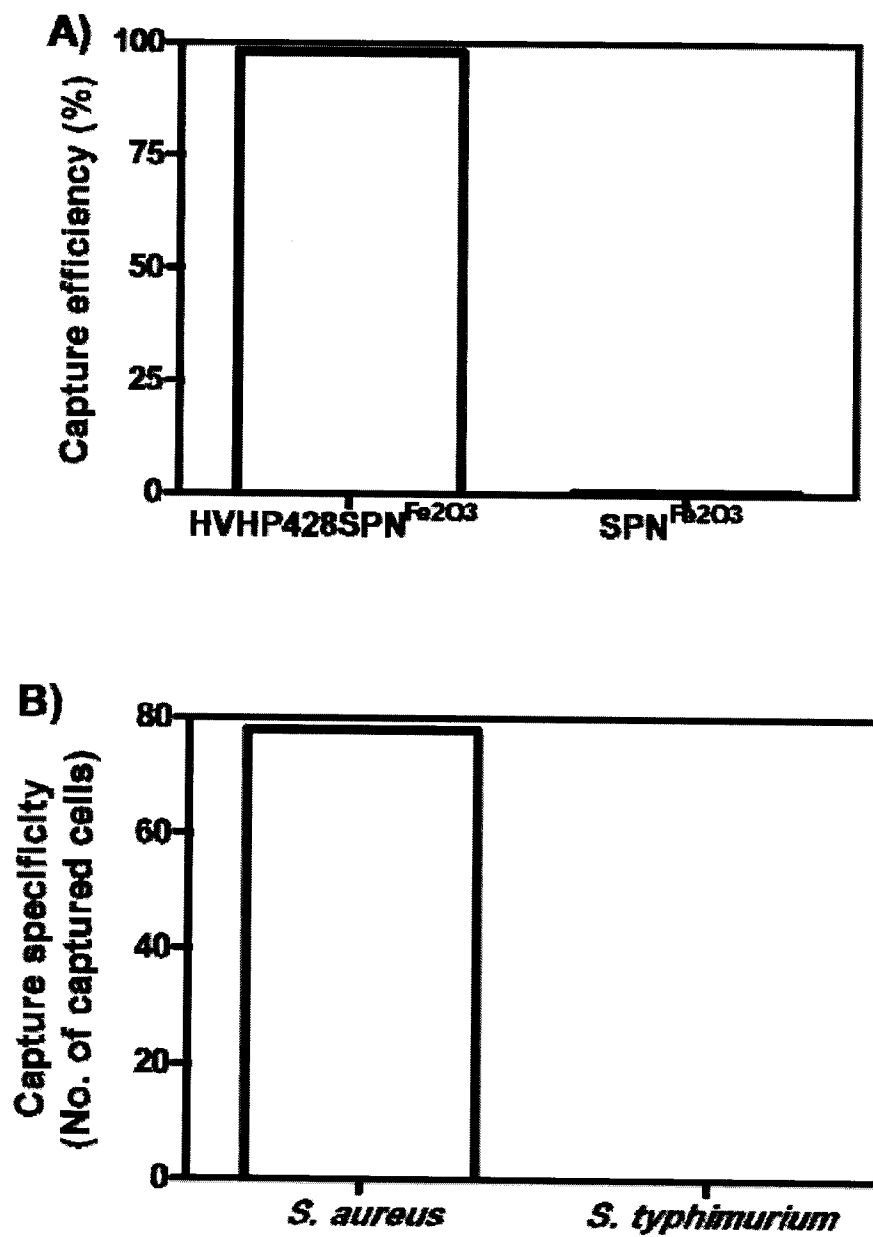


FIG. 7

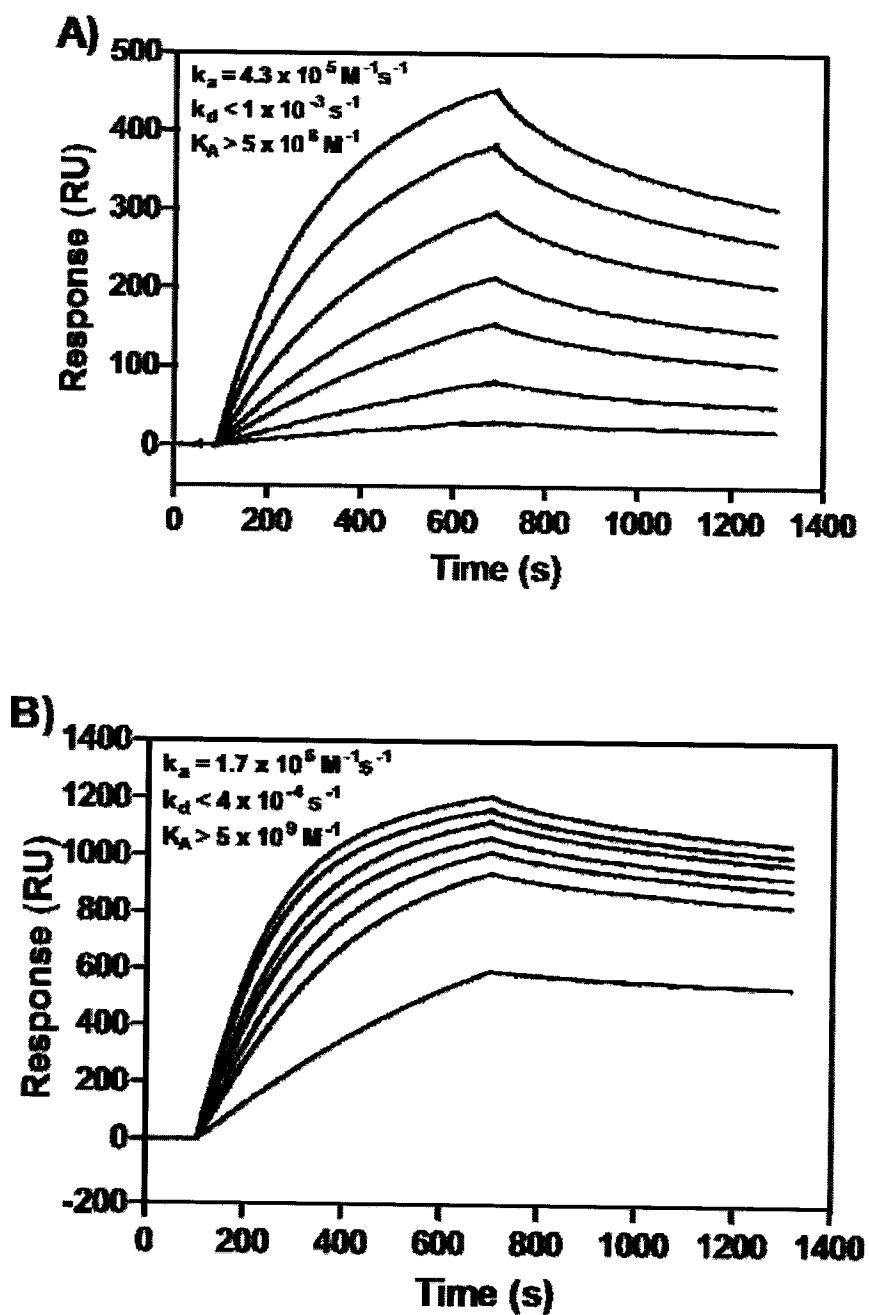


FIG. 8

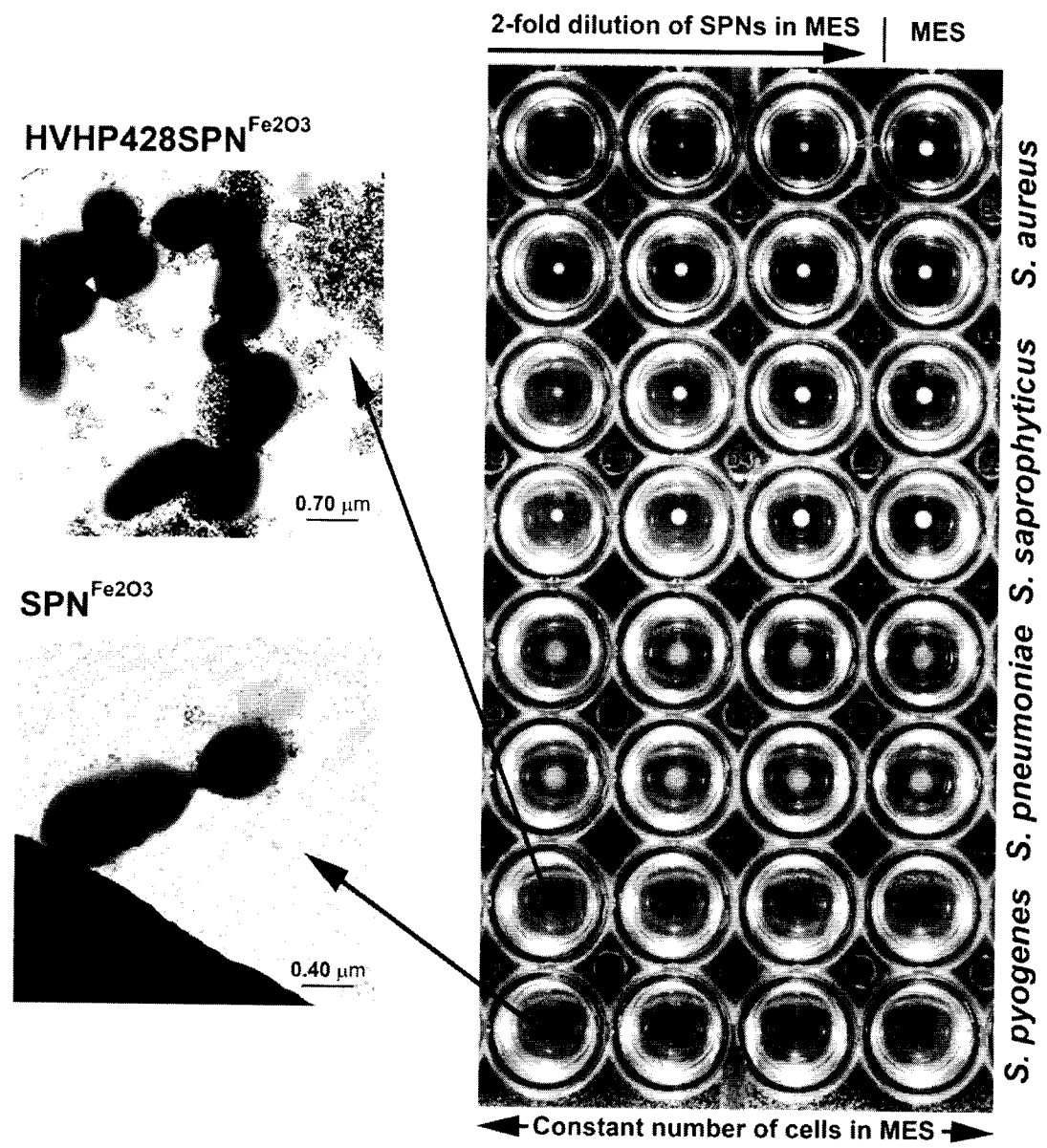


FIG. 9

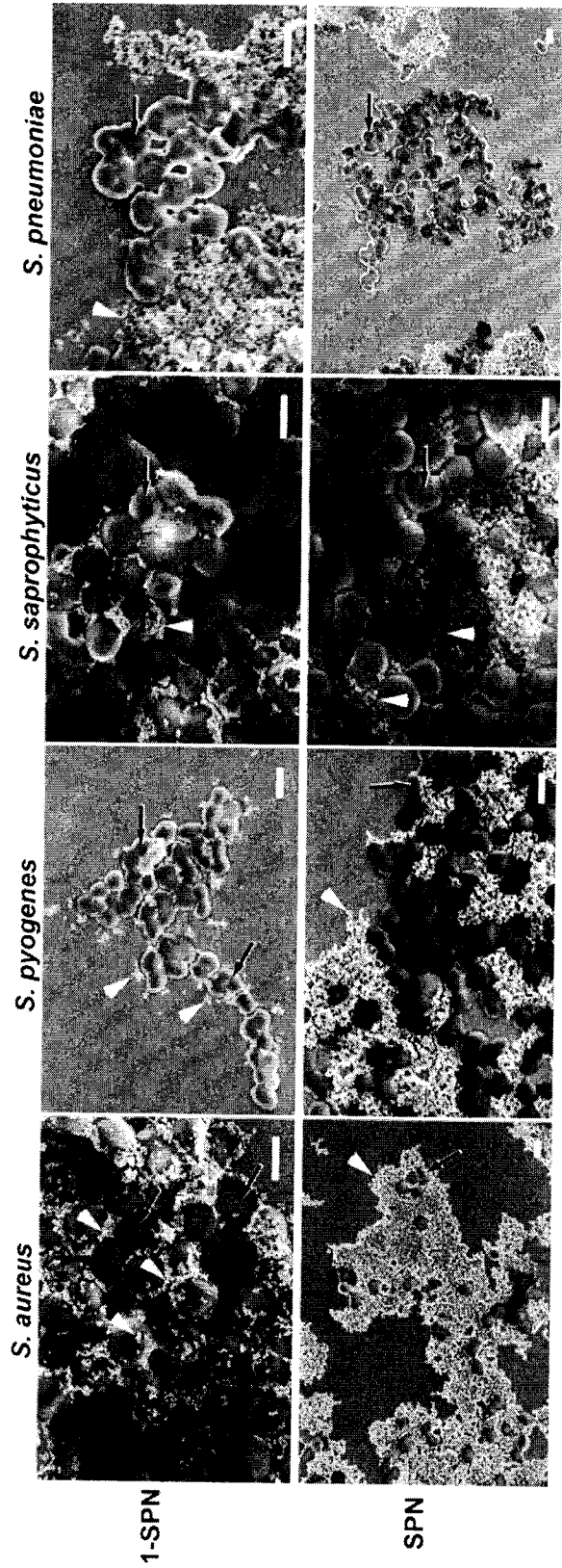


FIG. 10

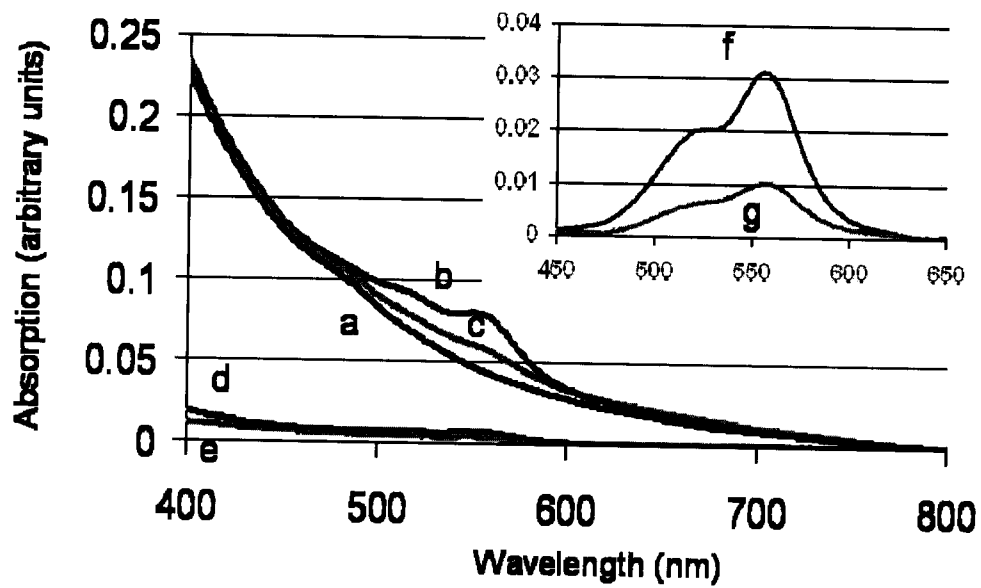


FIG. 11

Calibration curve for TMR in MES-buffered water

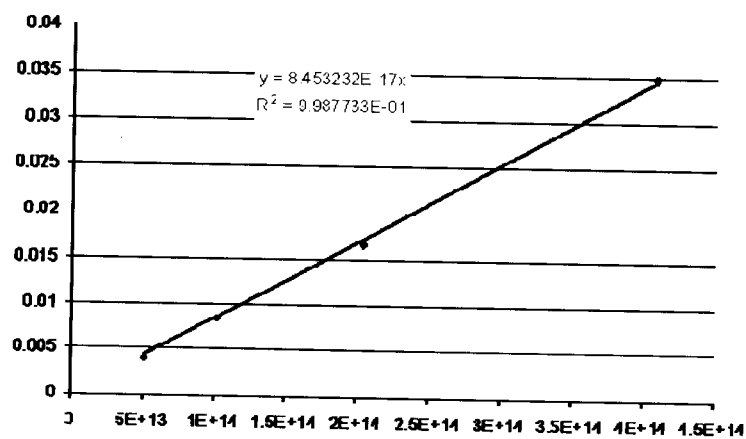


FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2009/001712

A. CLASSIFICATION OF SUBJECT MATTER IPC: <i>C07K 17/14</i> (2006.01), <i>C07K 16/00</i> (2006.01), <i>C07K 16/12</i> (2006.01), <i>C12N 1/00</i> (2006.01), <i>C12N 1/20</i> (2006.01), <i>C12N 5/071</i> (2010.01), <i>G01N 33/569</i> (2006.01) According to International Patent Classification (IPC) or to both national classification and IPC														
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C07K, C12N, G01N Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used) Databases: PubMed, Scopus, Delphion, Canadian Patent Database; Search terms: nanoparticle(s), magnetic, paramagnetic, superparamagnetic, beads, single domain, antibody, antibodies, inventors' names (in various combinations).														
C. DOCUMENTS CONSIDERED TO BE RELEVANT <table border="1"> <thead> <tr> <th>Category*</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td>X --- Y</td> <td>KELL, A.J. et al. "Multifunctional Nanoparticle Platforms for Pathogen Diagnostic Applications". In: Nanotechnology 2008: Technical Proceedings of the 2008 NSTI Nanotechnology Conference and Trade Show, Boston, June 1-5, 2008. Edited by NanoScience and Technology Inst. CRC press, 2008, Vol.2, pp 210-213, ISBN:978-1-4200-8504-4.</td> <td>1-3, 7-11 4-6</td> </tr> <tr> <td>Y</td> <td>LIANG, S. et al. Surface Modified Superparamagnetic Iron Oxide Nanoparticles: as a New Carrier for Bio-magnetically Targeted Therapy. J. Mater. Sci.: Mater. Med. 2007, Vol.18, No.12, pp2279-2302, ISSN: 0957-4530, the whole document.</td> <td>1-11</td> </tr> <tr> <td>Y</td> <td>TO, R. et al. Isolation of monomeric human V(H)s by a phage selection. J. Biol. Chem. 2005, Vol.280, No.50, pp 41395-41403, ISSN: 0021-9258, the whole document.</td> <td>1-11</td> </tr> </tbody> </table>			Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	X --- Y	KELL, A.J. et al. "Multifunctional Nanoparticle Platforms for Pathogen Diagnostic Applications". In: Nanotechnology 2008: Technical Proceedings of the 2008 NSTI Nanotechnology Conference and Trade Show, Boston, June 1-5, 2008. Edited by NanoScience and Technology Inst. CRC press, 2008, Vol.2, pp 210-213, ISBN:978-1-4200-8504-4.	1-3, 7-11 4-6	Y	LIANG, S. et al. Surface Modified Superparamagnetic Iron Oxide Nanoparticles: as a New Carrier for Bio-magnetically Targeted Therapy. J. Mater. Sci.: Mater. Med. 2007, Vol.18, No.12, pp2279-2302, ISSN: 0957-4530, the whole document.	1-11	Y	TO, R. et al. Isolation of monomeric human V(H)s by a phage selection. J. Biol. Chem. 2005, Vol.280, No.50, pp 41395-41403, ISSN: 0021-9258, the whole document.	1-11
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☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex. <table border="1"> <tbody> <tr> <td>* Special categories of cited documents :</td> <td>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"A" document defining the general state of the art which is not considered to be of particular relevance</td> <td>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"E" earlier application or patent but published on or after the international filing date</td> <td>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"&" document member of the same patent family</td> </tr> <tr> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td></td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </tbody> </table>			* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family	"O" document referring to an oral disclosure, use, exhibition or other means		"P" document published prior to the international filing date but later than the priority date claimed	
* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention													
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone													
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art													
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family													
"O" document referring to an oral disclosure, use, exhibition or other means														
"P" document published prior to the international filing date but later than the priority date claimed														
Date of the actual completion of the international search 14 January 2010 (14-01-2010)		Date of mailing of the international search report 8 February 2010 (08-02-2010)												
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476		Authorized officer Riad Qanbar (819) 934-7937												

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/CA2009/001712**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:
- a. (means)
- ☒ on paper
- ☒ in electronic form
- b. (time)
- ☒ in the international application as filed
- ☐ together with the international application in electronic form
- ☒ subsequently to this Authority for the purposes of search
2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments :