Supplementary Material (ESI) for Chemical Communications

Vancomycin Architecture Dependence on the Capture Efficiency of Antibody-modified Microbeads by Magnetic Nanoparticles

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Supplementary Information

Experimental

Water-soluble iron oxide nanoparticles (Fe_xO_y) were obtained from Ferrotec Corporation (average diameter 10 nm, EMG 304 ferrofluid, U.S.A.). Tetraethoxysilane (TEOS) and 3-aminopropyldiethoxymethylsilane (APDEMS) were both ordered from Gelest, Inc. Ammonium hydroxide (NH4-OH, 28-30 wt %), high purity 2-propanol, dimethylsulfoxide (DMSO), dimethylformamide (DMF) and dichloromethane were obtained from EMD Chemicals, Inc. Fluorescein cadaverine, 1μm FluoSphere carboxylate modified microspheres (365/415) and vancomycin-BODIPY® FL conjugate were purchased from Molecular Probes. Succinic anhydride, fluorescamine, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), diisopropylethylamine, N-hydroxy succinimide (NHS), 1-hydroxybenzotriazole (HOBT), *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), vancomycin, MES and PBS were purchased from Aldrich and used without further purification. Vancomycin polyclonal to vancomycin antibody (ab19968) was purchased from Abcam. Water was purified with a Millipore Q-guard 2 purification system (Millipore Corporation). Only purified water was used in the experiments.

The vancomycin derivative was purified by RP-preparative HPLC on a Waters Delta Prep 4000 system, equipped with a Waters 996 photodiode detector and a Zorbax 300SB-C18 column. Analytical RP-HPLC was performed with an Agilent 1100 series HPLC employing a Zorbax 300SB-C18 column (5μm) with dimensions of 4.6x150 mm.

Transmission electron microscopy (TEM) was used to characterize the various $Fe_xO_y@SiO_2$ based core-shell nanoparticles. The TEM images were obtained using a Philips CM20 FEG microscope operating at 200 kV. Samples were prepared by dropcasting several drops of the particle dispersion onto 200 mesh copper/Formvar Tem grids with a pipet.

The fluorescence emission spectra were recorded on a Fluoromax-3 spectrometer (Jobin Yvon Horiba, Instruments SA) with a 450 W Xe lamp as the excitation source and an excitation wavelength of 365 nm and spectra-recording at 380-600 nm.

Modification of the polystyrene beads with vancomycin antibody (vAB-PS)

Blue fluorescent (365/415) FluoSphere carboxylate modified microspheres (Molecular Probes) (1μm, 0.5 mL, $1.2x10^9$microspheres) were centrifuged and redispersed in 1.5 mL of 30mM MES buffer at pH=6. The microspheres were then mixed with 4 mg of rabbit polyclonal to vancomycin antibody (Abcam, ab19968) in 1 mL of MES buffer for 15 minutes before 30 mg of EDC was added to the mixture. Following gentle vortex mixing for 2 hours, the solution was centrifuged (2000 rpm, 10 minutes), the supernatant removed and the microspheres redispersed in fresh phosphate buffered saline (PBS) buffer, pH=7.5, four times. The microspheres were finally dispersed in 1.5 mL of PBS buffer, resulting in a concentration of ~$1.2x10^9$ **vAb-PS**/mL.

Synthesis of core-shell silicon dioxide encapsulated iron oxide nanoparticles (Fe_xO_y@SiO_2) (SNP)

The nanoparticles were synthesized as reported by Ma and coworkers[1] and Xia and coworkers.[2] Briefly, 0.4 mL of a commercially available ferrofluid (ferrotec EMD304, comprised of a mixture of Fe_3O_4 and Fe_2O_3 nanoparticles)[1, 2] was diluted in 400 mL of Millipore water. This stock solution was then further diluted (6 mL of the stock solution was diluted to 42 mL) with Millipore water and the solution was sonicated for 30 minutes. Following sonication, the solution of nanoparticles was transferred to a 3-necked 500 mL flask containing 400 mL isopropanol and mechanically stirred. Tetraethoxysilane (0.80 mL) was added to the flask and stirred for ~1 minute. Following 1 minute of stirring, 6 mL of 28% ammonium hydroxide was added all at once and stirring was continued overnight. Analysis of these nanoparticles by TEM shows that these conditions generate core-shell nanoparticles of a total diameter of 42±6 nm, consisting of a ~5-10 nm iron oxide core surrounded by a smooth silicon dioxide shell with a thickness of 15-20 nm. There are no detectable free Fe_xO_y nanoparticles or free silica nanoparticles. A TEM image of the nanoparticles is shown below in Figure S1.

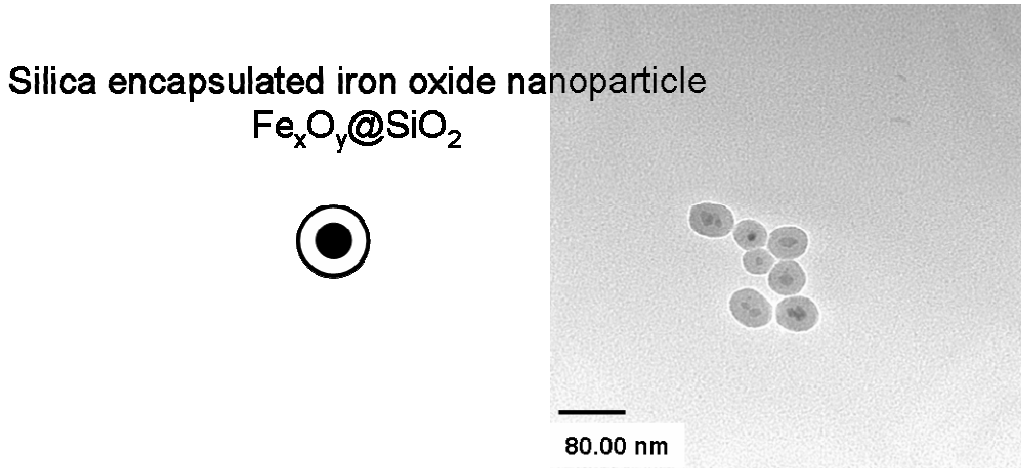

Figure S1: A TEM image of the $Fe_xO_y@SiO_2$ nanoparticles (**SNP**).

Surface modification of silicon dioxide encapsulated iron oxide nanoparticles (SNP)

Amine-modified silica encapsulated iron oxide nanoparticles (SNP-1)

The **SNP**s described above were equally distributed into 10 falcon tubes (~45mL/tube) and to six of the tubes 0.9 mL of aminopropyldiethoxymethylsilane (APDEMS) was added in order to modify the surface with a functional amine group. The mixture was shaken on a mechanical shaker for 18-24 hours at which time the tubes were centrifuged at 7000 rpm for 45 minutes in order to precipitate the nanoparticle. The nanoparticles were redispersed in DMF (15 mL) and centrifuged once again at 7000 rpm for 30 minutes to pellet **SNP-1** and separate it from free, unreacted APDEMS (process repeated three times). For experiments employing **SNP-1**, the nanoparticles were

dispersed in 30mM MES buffer at pH 6 and centrifuged (7000 rpm, 35 minutes) three times to ensure all DMF was removed. A TEM image of **SNP-1** dropcast from MES buffer is shown below in Figure S2. The number of amine groups anchored to the surface of the nanoparticle surface was quantified by comparing the fluorescence intensity from **SNP-1(fluorescamine)** nanoparticle with that of a calibration curve constructed from various concentrations of ethanolamine reacted with fluorescamine. Fluorescamine is a non-fluorescent substrate that reacts with primary amines to generate a fluorescent derivative. The reaction scheme is outlined in Figure S3. The experiment involved dispersing $1x10^{13}$ **SNP-1** in 1 mL of MES buffer and adding three drops of a 10mg/mL solution of fluorescamine in acetone to the nanoparticle solution. The nanoparticle was then magnetically confined to ensure that all of the fluorescence response was due to **SNP-1(Fluorescamine)** and not free APDEMS. This is easily determined as the fluorescene intensity decreases upon magnetic confinement, suggesting that there is very little free amine in the solution (i.e. the fluorescence intensity decreases >90% upon magnetic confinement of the nanoparticle). These data coupled with the calibration curve suggest that there are ~2500-3000 amines on each nanoparticle. It is also worth noting that **SNP-1** is stable in MES buffer for at least one month with little to no loss of amine functionality from the surface of the nanoparticle, as evidenced by reaction with fluorescamine one month after preparation (Figure S3).

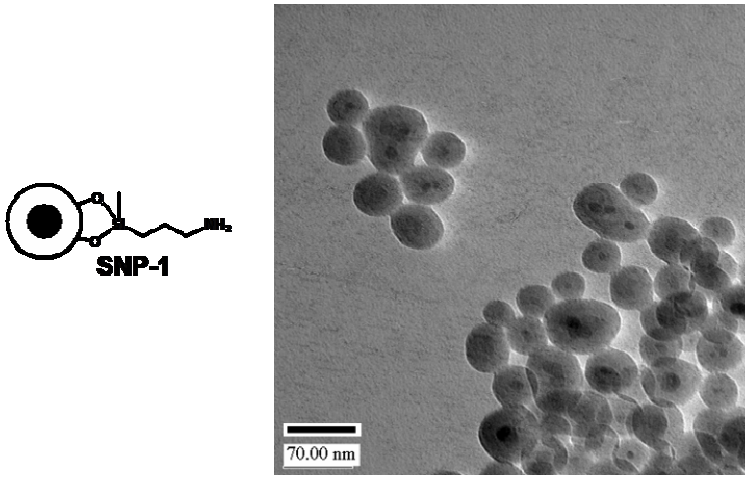

Figure S2: A TEM image of the amine-modified $Fe_xO_y@SiO_2$ nanoparticles (**SNP-1**).

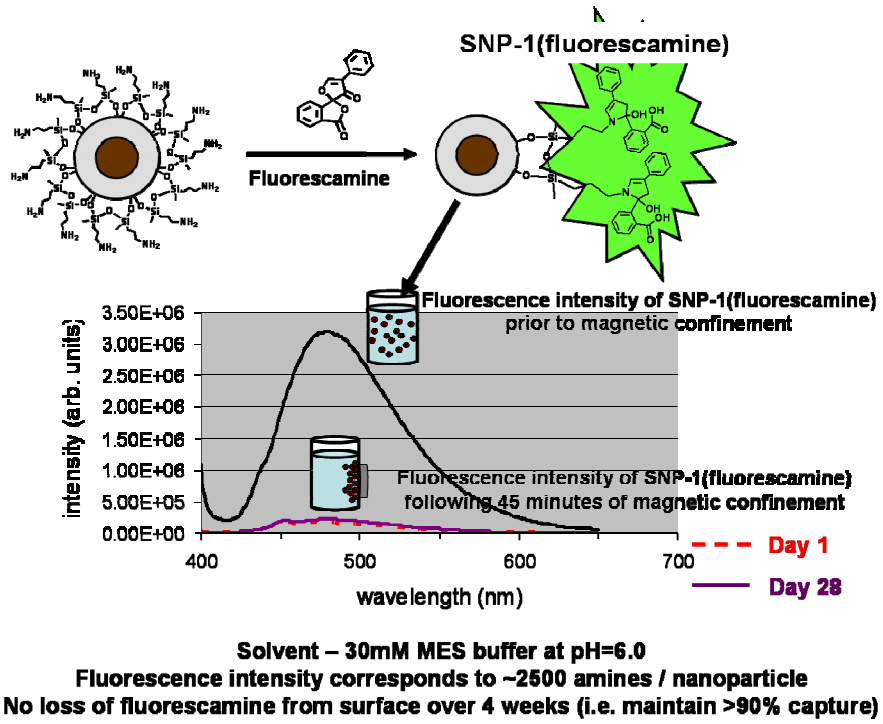

Figure S3: The stability and amine surface coverage of **SNP-1** by as determined by reaction with fluorescamine.

Carboxylate modified silica encapsulated iron oxide nanoparticles (SNP-2)

Carboxylate modified nanoparticles (**SNP-2**) are derived directly from **SNP-1**. Instead of dispersing the **SNP-1** nanoparticles in MES buffer, the nanoparticles were left in DMF and succinic anhydride was added to the tube to make a 1% solution and vortex mixed for 8 hours. The resulting nanoparticles were washed with fresh DMF several times to ensure all free, unreacted succinic anhydride had been removed. The nanoparticles were then dispersed in 30mM MES buffer (pH=6) and centrifuged and redispersed in fresh MES buffer several times to ensure all DMF was removed. The efficiency of the reaction was determined by adding fluorescamine (which would react with any remaining amine), which did not result in a fluorescence response. This suggests that at least 90% of the amine groups had reacted with the succinic anhydride. A TEM image of the nanoparticles is shown below in Figure S4.

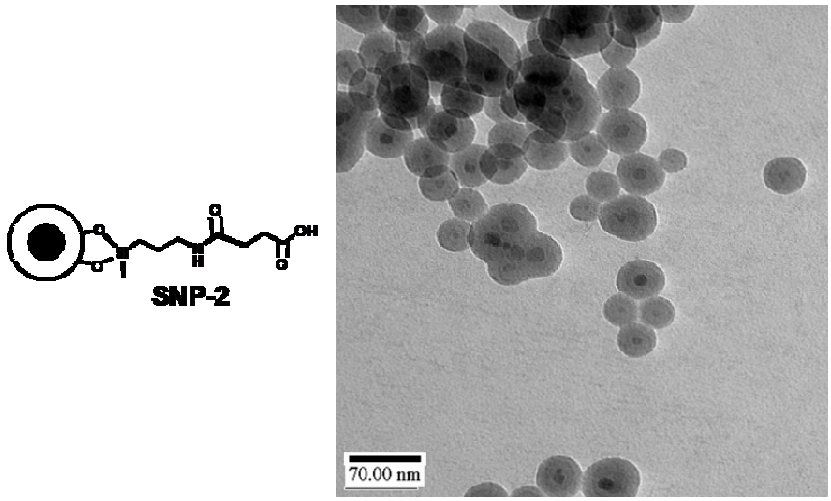

Figure S4: A TEM image of the carboxylic acid-modified $Fe_xO_y@SiO_2$ nanoparticles (**SNP-2**).

Modification of an amine-modified nanoparticle through the carboxylic acid moiety of vancomycin (SNP-3)

The amine-modified nanoparticles (**SNP-1**) in 18 mL ($1x10^{13}$ particles/mL) of 30 mM MES buffer was charged with 2 mg of vancomycin, 8 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 6 mg of N-hydroxysuccinimide (NHS) and mixed for 6 hours at room temperature. The resulting vancomycin-modified nanoparticles (**SNP-3**) were centrifuged and redispersed in fresh 30mM MES buffer (6 mL) 3 times to ensure all of the free, unreacted vancomycin, EDC and NHS were washed free of the nanoparticle. Following these wash steps, the **SNP-3** was dispersed in 16 mL of 30 mM MES and stored in the fridge at 4ºC. It is noteworthy to highlight that there is only one carboxylic acid present on the vancomycin molecule, hence we know the architecture of vancomycin on the nanoparticle surface. A TEM image of the nanoparticles is shown below in Figure S5.

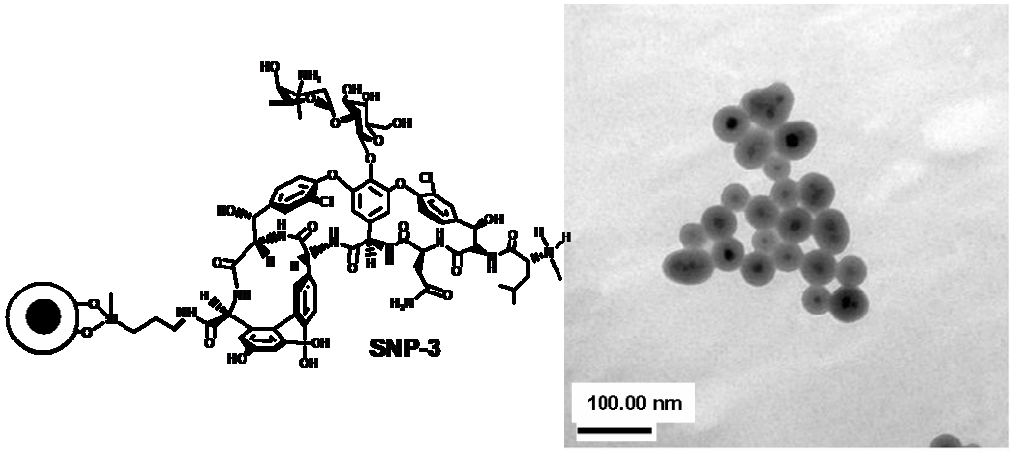

Figure S5: A TEM image of the vancomycin-modified $Fe_xO_y@SiO_2$ nanoparticles (**SNP-3**).

In order to estimate the quantitiy of vancomycin on **SNP-3**, a model reaction was carried out in which a BODIPY-modified vancomycin was reacted with **SNP-1**. Under conditions identical to those outlined above, 0.1 mg of BODIPY-vancomycin (Invitrogen) and 3 mL of **SNP-1** ($1x10^{13}$ particles/mL) in 30 mM MES buffer was charged with 1.5 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 1.0 mg of N-hydroxysuccinimide (NHS) and mixed for 6 hours at room temperature. The resulting nanoparticle was washed in an identical fashion to that described above. The number of vancomycin molecules anchored to the nanoparticle surface was quantified by comparing the fluorescence intensity from the **SNP-3**(**BODIPY**) with that of a calibration curve constructed from various concentrations of BODIPY-vancomycin. These data estimate there to be 9 vancomycin molecules on **SNP-3**. It should be noted that in the model reaction, the supernatant was highly fluorescent following centrifugation, suggesting that there was sufficient excess reagent to completely react with **SNP-1** (i.e. the **SNP-1** was the limiting reagent).

Anchoring vancomycin to the nanoparticle surface through the vancosamine amine moiety (SNP-4)

The carboxylate-modified nanoparticle (**SNP-2**) ($1x10^{13}$ particles/mL) in 8 mL of 30 mM MES buffer was charged with 2 mg EDC and 2 mg NHS and mixed for 30 minutes at room temperature. The resulting N-hydroxysuccinimidyl ester-modified nanoparticle (**SNP-2**(**NHS**)) was centrifuged and redispersed in fresh 30mM MES buffer (8 mL) 3 times to ensure all unreacted EDC and NHS were washed free of **SNP-2**. The solution was then charged with 2 mg of vancomycin and stirred for 3 hours at room

temperature. The resulting vancomycin-modified nanoparticle (**SNP-4**) was centrifuged and unreacted vancomycin was washed away with 8 mL MES buffer four times and finally dispersed in 8 mL MES and stored in the fridge at 4ºC. A TEM image of the nanoparticles is shown below in Figure S6.

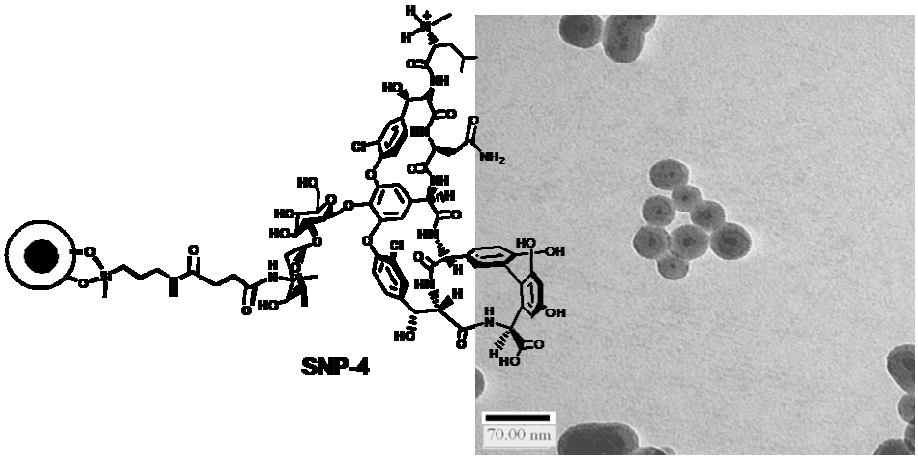

Figure S6: A TEM image of the vancomycin-modified $Fe_xO_y@SiO_2$ nanoparticles (**SNP-4**).

It is noteworthy to highlight that there are two amines on vancomycin; a primary nitrogen on the vancosamine sugar ring and a secondary amine on the N-methyl leucine residue. Based both on an investigation by Adamczyk[3, 4] and on a model reaction we have carried out employing similar reaction conditions to those employed in the preparation of the **SNP-4**, we confirmed that the vancomycin should be anchored to the surface of **SNP-4** through the vancosamine nitrogen and not the N-methyl leucine

residue. The can be ascertained through the use of mass spectrometry. Carrying out HPLC-MS analysis of the reaction products of the model reaction, we confirm that there are no peaks in the HPLC chromatogram that show a molecular ion that fragments with an unmodified vancosamine sugar or unmodified disaccharide, suggesting that the only site of modification is the vancosamine nitrogen. This data is pictured in Figure S7.

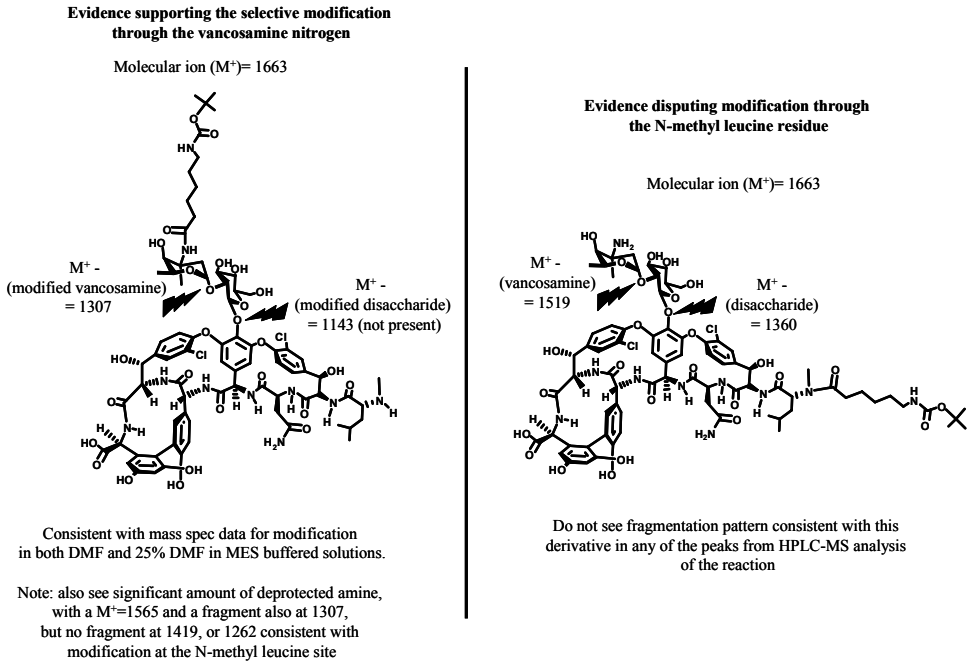

Figure S7: The HPLC-MS fragmentation products of the modified vancomycin model reaction.

Fluorescein-vancomcyin

Vancomycin (50 mg, $3.4x10^{-5}$ mol) was dissolved in 1 mL of DMSO. To this was added fluorescein cadaverine (24mg, $3.7x10^{-5}$ mol) in 1 mL DMF. The mixture was stirred and HBTU (19mg, $5.0x10^{-5}$ mol) and HOBT (6.6mg, $4.9x10^{-5}$ mol) were added as 0.25 mL solutions in DMF. Finally, diisopropylethylamine (10μL, $5.7x10^{-5}$ mol) was added and the mixture was stirred overnight. The product was precipitated with dichloromethane and centrifuged to isolate the orange/yellow product. The precipitate was dissolved in 3mL of Millipore water and purified via preparative reverse-phase HPLC (5-50% water:acetonitrile with no acid or buffer over 20 minutes, Rf=14.1 minutes). The product was characterized by HPLC (>93% by area) and ES-MS. The MS exhibited an ion of m/z=1922, consistent with a molecular ion of $[C_{92}H_{99}Cl_2N_{12}O_{28}S]^+$.

When this molecule had been prepared, it was used in an experiment to estimate the number of vancomycin molecules anchored to the surface of **SNP-4**. Under conditions identical to those applied above for the preparation of **SNP-4**, the carboxylate-modified nanoparticle (**SNP-2**) ($1x10^{13}$ particles/mL) in 5 mL of 30 mM MES buffer was charged with 1.5 mg EDC and 1.5 mg NHS and mixed for 30 minutes at room temperature. The resulting **SNP-2(NHS)** nanoparticle was centrifuged and redispersed in fresh 30mM MES buffer (5 mL) 3 times to ensure all unreacted EDC and NHS were washed free of the nanoparticle. The solution was then charged with 2 mg of fluorescein-vancomycin and stirred for 3 hours at room temperature. The resulting **SNP-4** (**fluorescein**) was washed in an identical fashion to that described above for **SNP-4**. The number of vancomycin molecules anchored to the nanoparticle surface was quantified by comparing the fluorescence intensity from the **SNP-4**(**fluorescein**) with that of a

calibration curve constructed from various concentrations of fluorescein-vancomycin. These data indicate there are 12 vancomycin molecules on each **SNP-4(fluorescein)** and hence we estimate the same number of vancomycin on **SNP-4**. It should be noted that in the model reaction, the supernatant was highly fluorescent following centrifugation, suggesting that there was sufficient excess reagent to completely react with **SNP-2** (i.e. the **SNP-2** was the limiting reagent).

Fluorescence experiments

In separate experiments, a 25μL aliquot ($3x10^7$ microspheres) of **vAb-PS** was added to 1mL ($5x10^{12}$ nanoparticles) of **SNP-3** and **SNP-4** in MES buffered water (pH=6) in a cuvette. A fluorescence emission spectrum of the sample was run immediately following the addition of the **vAb-PS** and then thirty minutes after the addition to ensure there is no non-specific absorption of the microspheres to the cuvette or any precipitation of the microsphere-nanoparticle conjugate from solution over this timescale which would result in a loss of fluorescence signal and could be interpreted as magnetic capture. We verified that neither of these instances occurs. A rare earth magnet was then placed at the side of the cuvette and the relative capture efficiencies of **SNP-3** and **SNP-4** were elucidated by monitoring how quickly the fluorescence intensity of the **vAb-PS** decreased as a result of its interaction with the superparamagnetic **SNP-3** and **SNP-4**. These data are pictured in Figure 2 of the manuscript. A typical experiment illustrating the differences observed in the fluorescence emission spectra prior to magnetic confinement (T=0 minutes) and following 10 and 15 and 30 minutes of magnetic confinement (T=10 (**SNP-3**) and T=15 and 30 minutes (**SNP-4**), respectively) are

depicted in Figure S8. TEM images of **SNP-3** and **SNP-4** interacting with **vAb-PS** are shown in Figures S9 and Figure S10, respectively.

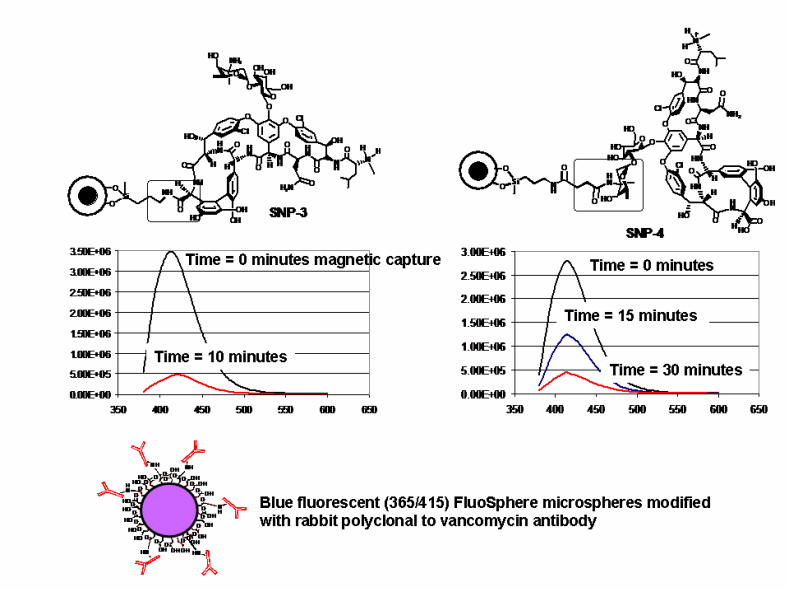

Figure S8: The fluorescence emission spectra of **SNP-3** and **SNP-4** mixed with **vAb-PS** prior to magnetic confinement (Time=0 minutes) and following magnetic confinement for 10 minutes (Time=10 minutes) for **SNP-3** and for 15 and 30 (Time=15 and Time=30 minutes, respectively) for **SNP-4**. The blue fluorescent FluorSphere polystyrene microbeads were irradiated at 365nm and the emission was monitored from 380-600nm.

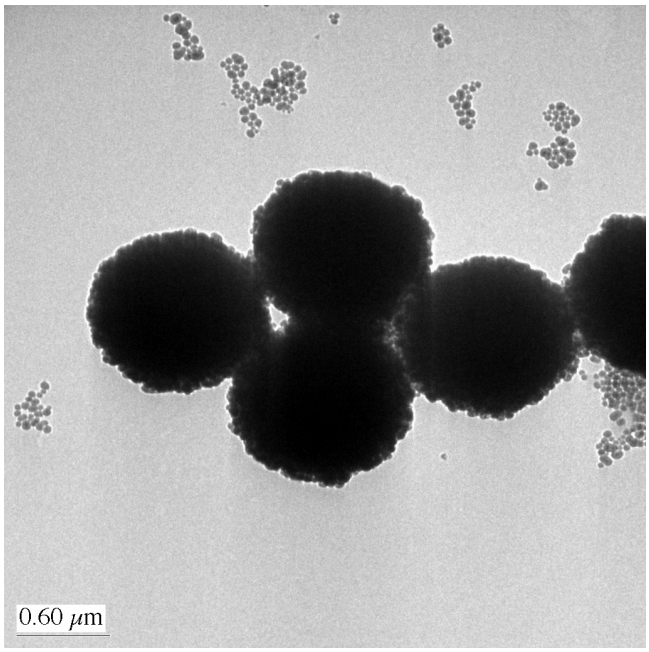

Figure S9: A TEM image of the **SNP-3-vAb-PS** conjugate.

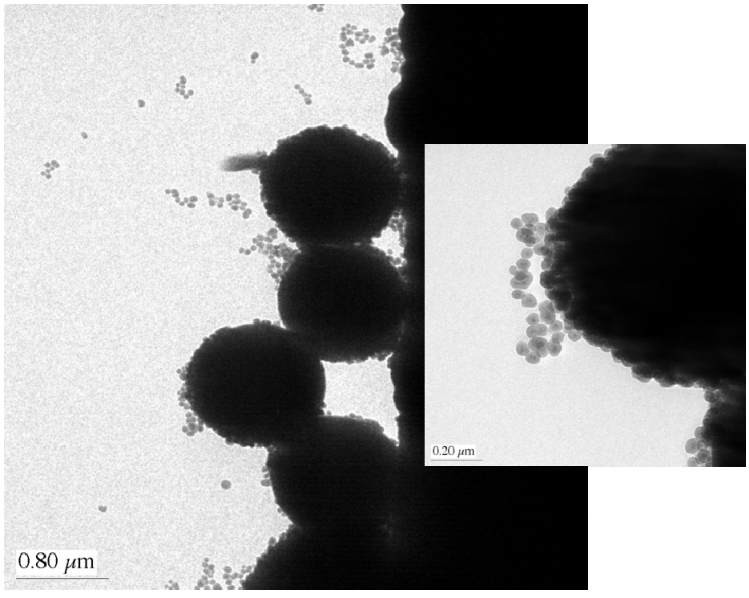

Figure S10: A TEM image of the **SNP-4-vAb-PS** conjugate

As a control experiment, 25 µL aliquots of **vAb-PS** ($3x10^7$ particles) were added to amine-modified (**SNP-1**) and carboxylate-modified (**SNP-2**) nanoparticles (1mL, $5x10^{12}$ nanoparticles) in 30mM MES buffered water to ensure that non-specific absorption of the **SNP**s was not contributing to the capture of **vAb-PS**. A typical experiment illustrating the differences observed in the fluorescence emission spectra prior to magnetic confinement (T=0 minutes) and following 10 and 30 minutes of magnetic confinement (T=10 (**SNP-1**) and T=30 minutes (**SNP-2**), respectively) are depicted in Figure S11. TEM images of **SNP-1** and **SNP-2** interacting with **vAb-PS** are shown in Figures S12 and Figure S13, respectively.

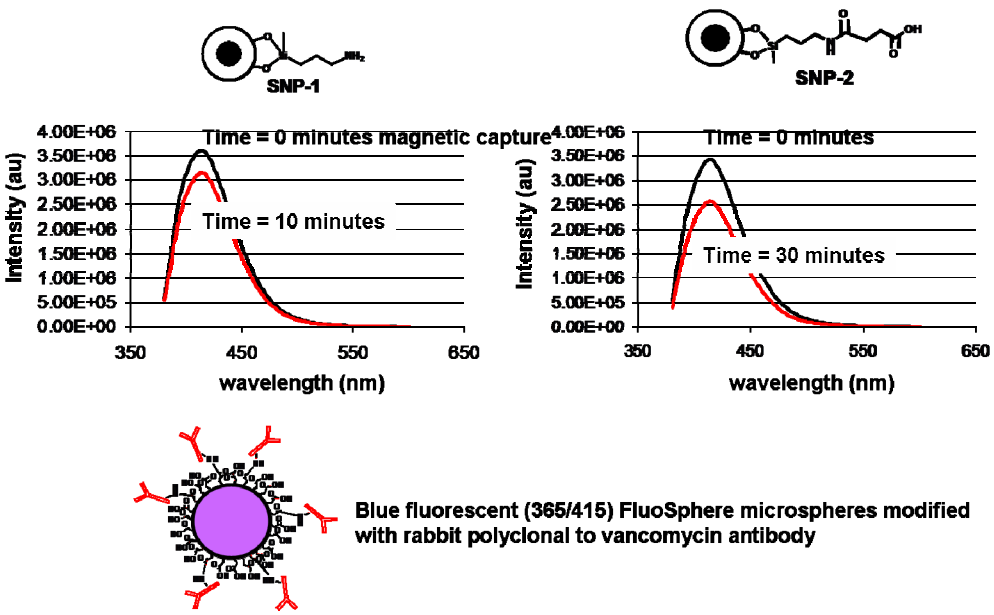

Figure S11: The fluorescence emission spectra of **vAb-PS** mixed with **SNP-1** and **SNP-2** prior to magnetic confinement (Time=0 minutes) and following magnetic confinement for 10 minutes (Time=10 minutes) for **SNP-1** and for 30 minutes (Time-30 minutes) for **SNP-2**. The blue fluorescent FluorSphere polystyrene microbeads were irradiated at 365nm and the emission was monitored from 380-600nm.

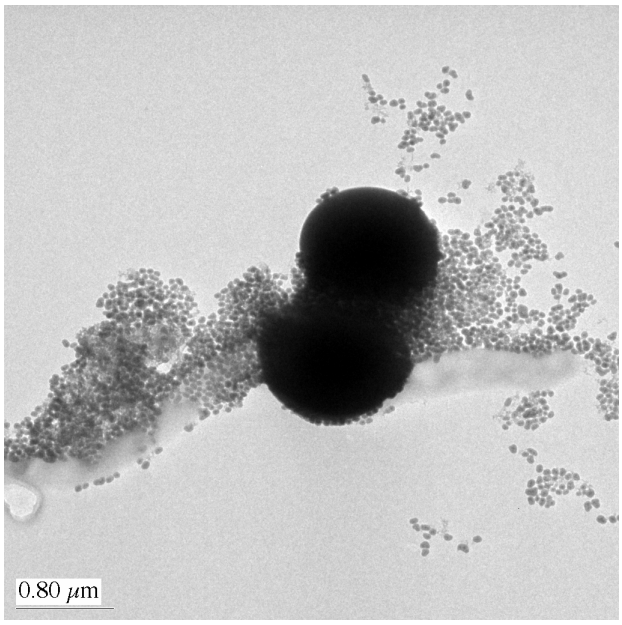

Figure S12: A TEM image of the **SNP-1** mixed with **vAb-PS**.

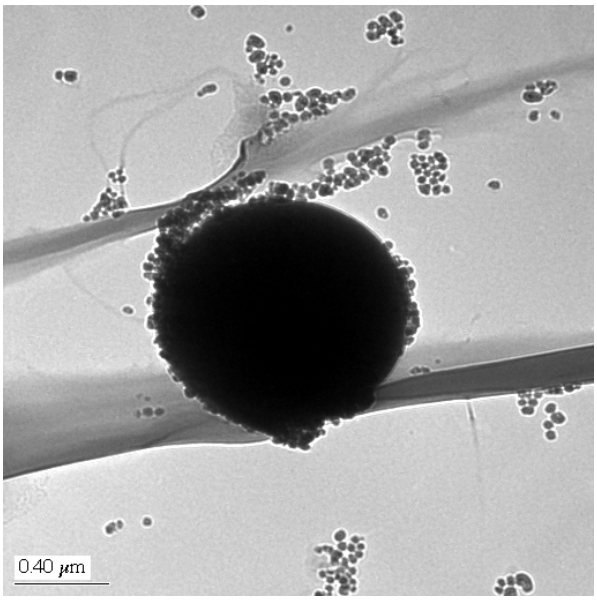

Figure S13: A TEM image of the **SNP-2** mixed with **vAb-PS**.

1. Ma, D.; Guan, J.; Normadin, F.; Denommee, S.; Enright, G.; Veres, T.; Simard, B., *Chem. Mater.* **2006,** 18, 1920-1927.
2. Lu, Y.; Yin, Y.; Mayers, B. T.; Xia, Y., *Nano. Lett.* **2002,** 2, 183 - 186.
3. Adamczyk, M.; Grote, J.; Moore, J. A.; Rege, S. D.; Yu, Z., *Bioconjugate Chem.* **1999,** 10, 176-185.
4. Adamczyk, M.; Moore, J. A.; Rege, S. D.; Yu, Z., *Bioorg. Med. Chem. Lett.* **1999,** 9, 2437-2440.