Detection of pathogenic *Streptococcus suis* bacteria using magnetic glycoparticles†

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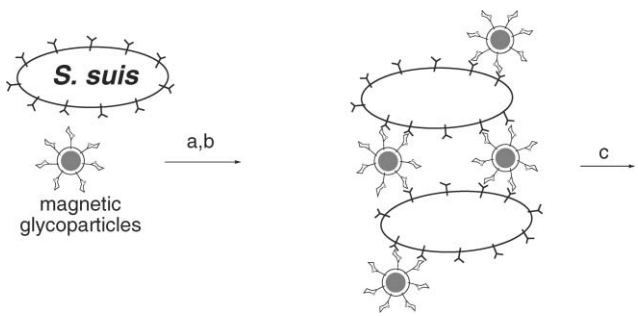
Detection of the zoonotic bacterial pathogen *Streptococcus suis* was achieved using magnetic glycoparticles. The bacteria contain an adhesion protein for the carbohydrate sequence Galα1,4Gal. After incubation with various amounts of the pathogen, magnetic concentration and ATP detection, bacterial levels down to 10^5 cfu could be detected. Submicrometer particles were needed, since with the larger microparticles the method did not succeed.

Introduction

There is an urgent need to rapidly identify a bacterial pathogen in case of a serious infection, in order to make a treatment plan. The rapid identification and characterization of bacterial pathogens is an important goal to serve as an alternative to time-consuming culturing. Bacteria carry adhesion proteins on their surface and these are crucial to the infection process.[1,2,3] The binding specificity of bacteria is a useful handle on the outside of the pathogen that can be taken advantage of in this context. Until now, experiments have focused on the detection of type 1 fimbriated uropathogenic *E. coli*, particularly because mannoside specific binding of the fimH adhesin is well established.[4]

In one such report, the detection was based on a fluorescent poly(*p*-phenylene ethynylene) polymer to which mannosides were coupled.[5] Detection of 10^4 cfu was possible by fluorescence microscopy after removing excess polymer by centrifugation and resuspending the (fluorescent) bacteria. In another approach a microarray displaying various monosaccharides was used.[6] The bacteria were made fluorescent with a cell-permeable nucleic acid staining fluorescent dye and specific binding to mannosides was observed. Silica-coated magnetite iron oxide nanoparticles with an average diameter of 10 nm[7] and larger micrometer and submicrometer-sized magnetic particles, as well as non-magnetic particles such as quantum dots[8] and gold nanoparticles,[9] were also used but only for *E. coli* detection.[10,11]

For the adhesion-based detection to become a practical method, it is essential that the method is extended to other species and other carbohydrate specificities. We here explore the magnetic glycoparticle-based bacterial detection for the first time on a gram-positive pathogen, *i.e.* the zoonotic *Streptococcus suis*.[12] This pathogen can cause meningitis, septicemia, and pneumonia in pigs and also meningitis in humans,[13,14,15] as such it is considered an emerging human threat.[16] It binds to the disaccharide galabiose (Galα1,4Gal) and was previously shown to bind much stronger to multivalent versions thereof, such as tetra- and octavalent glycodendrimers in solution.[17,18] The adhesin responsible for the galabiose binding has not yet been characterized and the number of adhesin molecules per bacteria is also unknown. The glycoparticles used were of 250 nm diameter and contained streptavidin conjugated on their surface. For this reason a biotinylated carbohydrate sequence was prepared. Thus, the particle preparation is relatively straightforward. The bacteria were incubated with the particles and after magnetic separation they were quantified by a standard luminescence-based ATP detection system (Scheme 1). Both monovalent and tetravalent galabiosides were used and monovalent GlcNAc was used as a negative control. We also explored larger ($d = 10\,\mu m$) particles to identify the possible benefit of the submicrometer ($d = 250$ nm) dimensions.



Scheme 1 Schematic display of the glycoglycoparticle-based *S. suis* detection method. a) incubate; b) magnetic separation; c) luminecent ATP detection.

Results and discussion

The synthesis started with the coupling of pentynoic acid **1** and the monoprotected diamine **2** (Scheme 2). After the 'click' coupling

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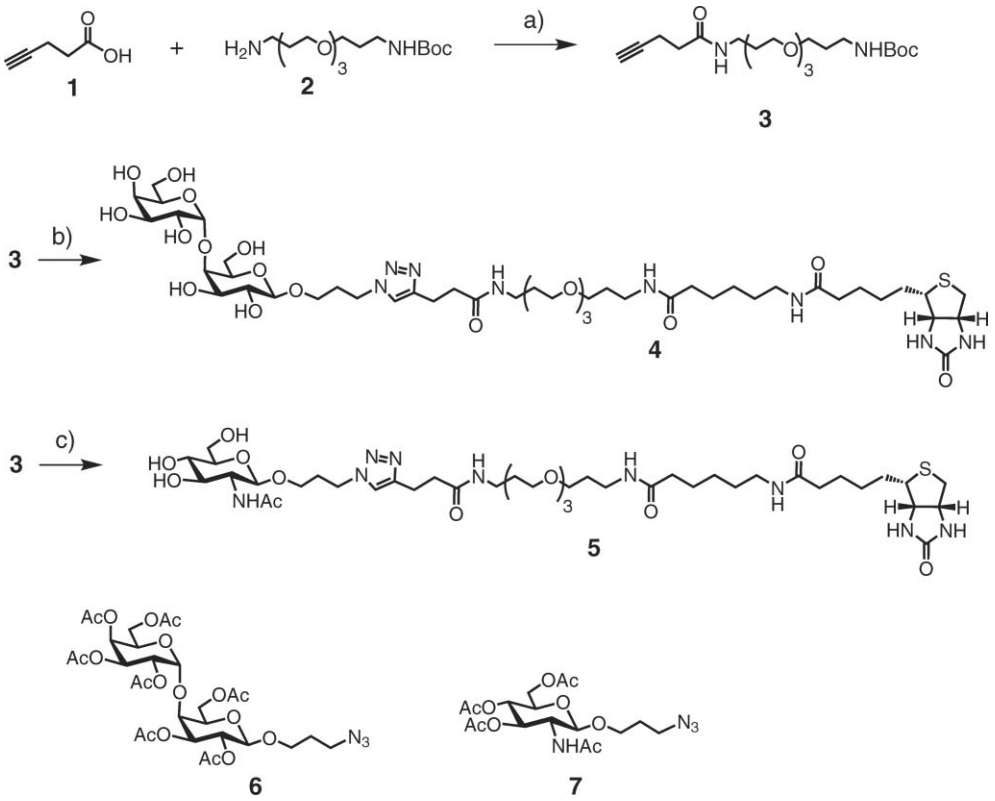


Scheme 2 a) DiPEA, BOP, 65%; b) i. **6**, $CuSO_4$, sodium ascorbate, DMF–H_2O, 90%; ii. NaOMe, MeOH, quant.; iii. TFA, quant. iv. aminocaproyl biotin NHS ester, 47% c) i. **7**, $CuSO_4$, sodium ascorbate, DMF–H_2O, 90%; ii. NaOMe, MeOH, quant.; iii. TFA, quant.; iv. aminocaproyl biotin NHS ester, 68%.

of galabiose building block **6**,[18] amine and hydroxyl deprotection steps, a biotin moiety was coupled to yield the desired **4**. A similar synthetic strategy yielded the GlcNAc derivative **5**. A tetravalent galabiose-linked compound was prepared from tetravalent dendrimer **8**.[18] First, the core methyl ester was saponified and the resulting carboxylic acid group was coupled to the monoprotected amine **2** (Scheme 3). Then the galabiose building block **6** was coupled to the exterior alkynes *via* 'click' chemistry. After amine and hydroxyl deprotection steps, a biotin moiety was coupled to yield the desired **10**.

The glycoparticles[19] were incubated with the biotinylated carbohydrates **4**, **5** and **10**. After this they were incubated with various dilutions of an overnight culture of *S. suis* D282 for an hour, and after magnetic concentration of the particles with attached bacteria and washing steps, the amount of added bacteria was determined with a luminescence assay that quantifies the bacterial ATP.[20] In order to compare different experiments the luminescence was normalized and results are displayed in Fig. 1. A clear signal, dependent on the amount of added bacteria, was obtained for both the monovalent **4** and the tetravalent **10**. In contrast, the experiments with the control GlcNAc conjugated particles **5** yielded only very minor signals, an indication that the galabiose recognition structure is required to obtain a significant signal. The detection limit as seen in Fig. 1 for **4** is around an OD of *S. suis* of 0.0005, which was determined to be of the order of 10^4 bacteria per mL. Interestingly, the monovalent **4** yielded stronger signals than the tetravalent **10**. In previous inhibition studies in solution tetravalent structures were shown to be far more effective

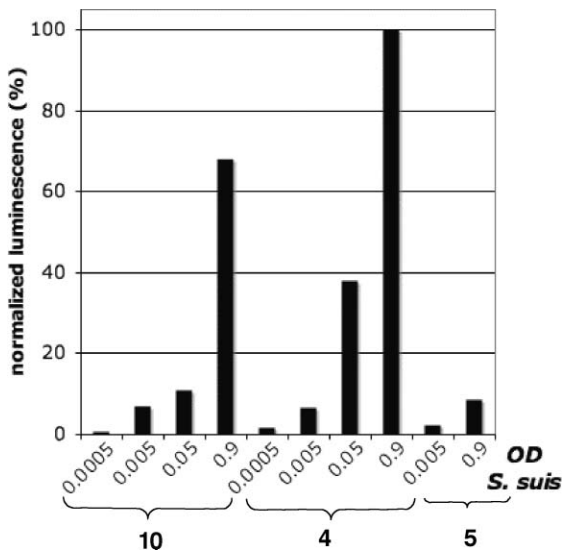
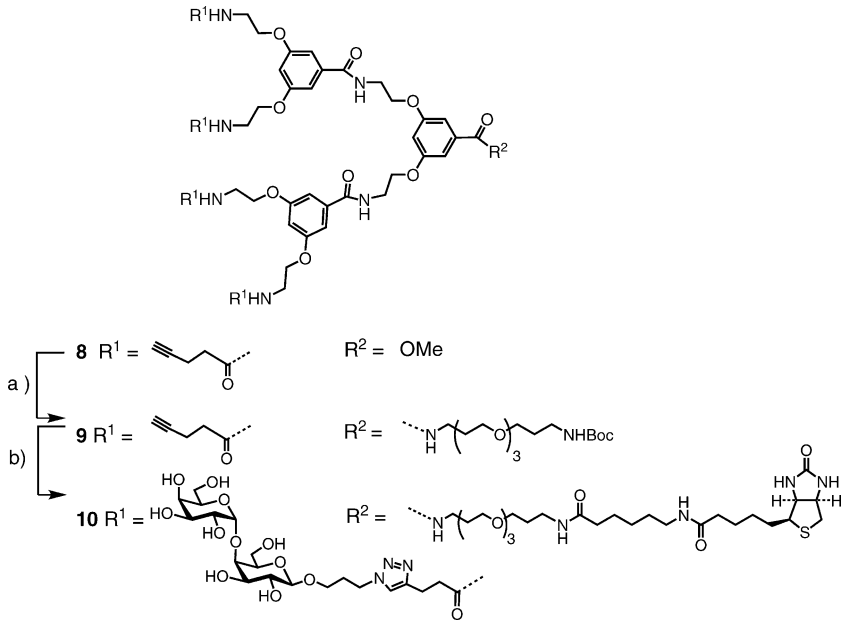


Fig. 1 Normalized luminescence signals obtained for the detection of *S. suis* D282 by magnetic glycoparticles displaying galabiose (**10**, **4**) and negative control GlcNAc (**5**).

inhibitors than monovalent ones.[17,18] This can be explained by considering the different types of multivalency effects.[3,21] The glycoparticle offers a multivalent presentation of either **4** or **10**, which, considering the 250 nm diameter of the (unconjugated) particles, could lead to multiple simultaneous interactions with



Scheme 3 a) i. NaOH, H_2O, MeOH, dioxane quant. ii. **2**, DiPEA, BOP, 72%; b) i. **6**, $CuSO_4$, sodium ascorbate, DMF–H_2O, 65%; ii. NaOMe, MeOH, quant.; iii. TFA, quant.; iv. aminocaproyl biotin NHS ester, 49%.

multiple copies of the adhesion protein on the bacterial surface. This type of multivalency effect apparently obscures or overrides the previously observed enhanced binding of a single tetravalent entity such as **10** to a single adhesin (aggregate), see ESI,† and represents one of only very few examples of this kind with bacteria.[22] By varying some of the parameters in the detection experiments (incubation time, coating concentration, particle size, amount of bacteria) it became clear which aspects are of crucial importance for a successful detection. Particularly instructive were experiments with larger magnetic particles with a diameter of 10 μm which did not enable successful bacterial detection. This indicates that the much larger surface area of the smaller glycoparticles with a 250 nm diameter is of crucial importance for successful bacterial detection.

Conclusions

Successful specific detection of *S. suis* was achieved using a relatively straightforward approach involving glycoparticles, a synthesized biotinylated sugar and an ATP detection system. The detection was possible due to a specific combination of features, being 1) The submicrometer-sized nature of particles; 2) the judicious choice of the accessible adhesin target molecule on the bacterial surface and 3) an adequate detection method of bound bacteria. This represents the first example of the detection of a gram-positive pathogen and in fact of a pathogen other than the uropathogenic *E. coli*. Considering the large losses of piglets due to *S. suis* in the pig farming business, a straightforward *S. suis* detection system is highly needed.[15] Remaining challenges in furthering the adhesion-based detection/characterization method is that multiple sugar specificities need to be used. Furthermore the variability of the detection due to phase variation[23] needs to be taken into account. Progress towards these goals will be reported in due course.

Experimental

Unless stated otherwise, chemicals were obtained from commercial sources and were used without further purification. Solvents were purchased from Biosolve (Valkenswaard, The Netherlands). Microwave reactions were carried out in a microwave Biotage Initiator (Uppsala, Sweden). The microwave power was limited by temperature control once the desired temperature was reached. A sealed vessel of 2–5 mL was used. Analytical HPLC runs were performed on a Shimadzu automated HPLC system with a reversed-phase column (Alltech, Adsorbosphere C8, 90 M, 5 mm, 250L4.6 mm, Deerfield, IL, USA) that was equipped with an evaporative light-scattering detector (PLELS 1000, Polymer Laboratories, Amherst, MA, USA) and a UV/Vis detector that was operating at 220 and 254 nm. Preparative HPLC runs were performed on a Applied Biosystems workstation. Elution was effected by using a linear gradient of 5% MeCN/0.1% TFA in H_2O to 5% H_2O/0.1% TFA in MeCN. ^1H NMR (300 MHz) and ^{13}C NMR (75.5 MHz) were performed on a Varian G-300 spectrometer. HSQC NMR (500 MHz) were performed on a VARIAN INOVA-500 spectrometer. Electrospray mass experiments were performed in a Shizmadu LCMS QP-8000. MALDI-TOF experiments were run in MALDI-TOF Kratos Shimadzu Axima-CFR using α-Cyano-4-hydroxycinnamic acid for calibration.

General "click" conjugation conditions

Alkyne dendrimer, sugar azide (1.5 equiv./alkyne), $CuSO_4$ (0.15 equiv./alkyne) and sodium ascorbate (0.3 equiv/alkyne) were dissolved in an appropriate volume of 1% H_2O in DMF. The mixture was heated under microwave irradiation to 80 °C for 30 min. The mixture was concentrated *in vacuo*, and the product was isolated by silica gel chromatography.

General deprotection procedure

Compounds were dissolved in MeOH whereupon catalytic NaOMe was added. The reaction mixture was stirred for 2 h and afterwards neutralized with Dowex H^+, filtered, and concentrated *in vacuo*. The residue was stirred in a solution of 5% H_2O in TFA for 1 h. Solvents were evaporated and the product was purified by preparative HPLC and lyophilized from H_2O–MeCN.

Monovalent alkyne 3

A solution of the spacer **2**[24] (160 mg, 0.5 mmol) was dissolved in CH_2Cl_2 whereupon pentynoic acid **1** (73.5 mg, 0.75 mmol), $^iPr_2EtN$ (0.09 mL, 0.5 mmol) and BOP (331.7 mg, 0.75 mmol) were added. The reaction was stirred for 18 h and followed by TLC (CH_2Cl_2: MeOH 9 : 1). After, the reaction mixture was taken up in EtOAc (100 mL) and washed twice with H_2O (50 mL) and brine (50 mL). The organic phase was dried with Na_2SO_4, filtered and concentrated. The crude product was purified by silica column chromatography to give **3** (130 mg, 65%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.3 (s, 1H, NH), 4.9 (s, 1H, NH), 3.6 (m, 12H, CH_2), 3.3 (q, J = 6.0, 2H, CH_2), 3.2 (q, J = 6.0, 2H, CH_2), 2.5 (m, 2H, CH_2), 2.3 (t, J = 7.1, 2H), 1.9 (q, J = 3.0, 1H, CH), 1.7 (q, J = 6.0, 4H, CH_2), 1.4 (s, 9H, CH_3). ^{13}CNMR (75.5 MHz, $CDCl_3$): δ = 171.4, (C(O)NH), 156.4 (NHC(O)C (CH3)3), 83.30 (CHαC), 79.17 (CHBoc), 70.49, 70.20, 70.09, 69.81, 69.45, 69.30 (CH_2O and CαCH), 38.36, 37.72 (CH_2NH), 35.36 (CH_2CO), 29.84, 29.08, 28.58(CH_3, Boc), 5.03(CH_2). ESMS for $C_{20}H_{36}N_2O_6$ (calcd. [M+Na]$^+$: 423.3): found [M+Na]$^+$ 423.5.

Biotinylated monovalent galabiose 4

A "Click" reaction with galabiose derivative **6** and alkyne **3** was performed by the general procedure. The product was isolated by silica gel chromatography (EtOAc–MeOH, 1 : 0–8 : 2) (125 mg, 90%). The deprotection was performed by the general procedure. The product was isolated and purified by preparative HPLC (CH_3CN–H_2O), (36.3 mg, 95%). ^{1}H NMR (300 MHz, D_2O) δ 7.7 (s, 1H, $CH_{triazole}$), 4.8 (s, 1H), 4.71 (s, 1H), 4.7–4.6 (m, 15H), 4.5 (s, 1H), 4.4 (t, J = 6.3, 2H), 4.3 (q, J = 8.0, 2H), 3.9 (s, 2H), 3.8–3.6 (m, 5H), 3.6–3.4 (m, 15H), 3.3 (t, J = 6.0, 2H), 3.0 (t, J = 6.8, 2H), 2.9 (t, J = 6.8, 2H), 2.8 (t, J = 6.8, 2H), 2.5 (t, J = 7.1, 2H), 2.0 (t, J = 6.0, 2H), 1.8 (t, J = 6.3, 2H), 1.5 (t, J = 6.3, 2H). ^{13}CNMR (from HSQC, D_2O): δ = 126.70, 105.40 (C-1), 103.32 (C-1′), 79.70, 77.80, 75.28, 73.62, 73.17, 72.92, 71.91, 71.72, 71.48, 71.42, 71.01, 70.85, 70.64, 69.17, 68.97, 62.85, 62.22, 49.36 ($CH_2N_{triazole}$), 40.31, 38.62, 37.36, 32.30, 30.19, 28.72, 23.45. MALDI-TOF for $C_{30}H_{55}N_5O_{15}$ (calcd. [M+Na]$^+$: 750.4): found [M+Na]$^+$ 750.6. A solution of the intermediate (25 mg, 0.030 mmol) was dissolved in dry DMF (2 mL) under N_2 atmosphere. The solution was basified with $^iPr_2EtN$ until pH 8 whereupon succinimidyl-6-(biotinamido)hexanoic acid (20.6 mg, 0.045 mmol) was added. The mixture was stirred for 72 h and monitored by TLC (EtOAc, MeOH, H_2O 6 : 3 : 1). Crude product was concentrated and purified by HPLC to give the compound **4** (15 mg, 47%). ^{1}H NMR (300 MHz, D_2O): δ = 7.70 (s, 1H, $H_{triazole}$), 4.83 (d, J = 3.5, 1H), 4.47 (s, 1H), 4.59 (s, 1H), 4.51–4.41 (m, 1H), 4.40–4.38 (m, 1H), 4.30–4.26 (m, 3H), 3.91 (s, 2H), 3.77–3.71 (m, 5H), 3.61–3.40 (m, 15H), 3.30 (t, J = 6.6, 2H), 3.25–3.15 (m, 1H), 3.11–3.04 (m, 6H), 2.79–2.85 (m, 3H), 2.66 (d, J = 9, 1H), 2.46 (t, J = 7.2, 2H), 2.09–2.07 (m, 6H), 1.66–1.16 (m,16H). ^{13}C NMR (from HSQC, D_2O): δ = 127.2, 105.6, 103.4, 80.62, 80.31, 79.99, 78.43, 75.3, 73.74, 73.75, 72.18, 71.87, 71.55, 71.50, 71.24, 70.93, 69.37, 69.05, 64.68, 63.12, 63.11, 62.8, 57.8, 49.99, 42.48, 42.17, 41.55, 39.36, 38.4, 37.8, 32.48, 30.92, 30.29, 28.11, 27.79, 23.42. ESMS for $C_{46}H_{80}N_8O_{13}S$ (calcd. [M+Na]$^+$: 1089.2): found [M+Na]$^+$ 1090.4.

Biotinylated GlcNAc 5

A "Click" reaction with GlcNAc derivative **7** and alkyne **3** was performed by the general procedure. The product was isolated by silica gel chromatography (CH_2Cl_2–MeOH, 19/1 → 9/1) (52 mg, quantitative). A deprotection reaction was performed by the general procedure. The deprotected product (33 mg, 0.055 mmol) was dissolved in dry DMF (2 mL) under N_2 atmosphere. The solution was basified with $^iPr_2EtN$ until pH 8 whereupon succinimidyl-6-(biotinamido)hexanoic acid (20.1 mg, 0.044 mmol) was added. The mixture was stirred for 72 h and monitored by TLC (EtOAc, MeOH, H_2O 6 : 3 : 1). Crude product was concentrated and purified by HPLC to give the compound **5** (35 mg, 68%). ^{1}H NMR (500 MHz, DMSO): δ = 7.83 (s, 1H, NH), 7.77 (s, 1H, NH), 7.72 (bs, 2H, NH, $H_{triazole}$), 4.30 (m, 3H), 4.13 (s, 1H, H1), 3.68 (d, J = 10.9, 2H), 3.48 (m, 6H), 3.38 (m, 4H), 3.30 (t, J = 7.6, 1H), 3.07 (m, 5H), 3.05 (s, 1H), 3.00 (s, 1H), 2.82 (t, J = 6.4, 2H), 2.57 (d, J = 12, 1H), 2.40 (t, J = 7.6, 1H), 2.04 (d, J = 5.2, 3H), 1.97 (m, 1H), 1.82 (s, 2H), 1.60 (d, J = 5.7, 4H), 1.46 (m, 4H), 1.37 (t, J = 7.0, 2H), 1.34 (m, 2H), 1.31 (m, 2H). ^{13}C NMR (from HSQC, DMSO): δ = 126.0, 104.7, 80.49, 77.67, 74.23, 73.92, 73.3, 72.98, 71.58, 68.45, 64.39, 62.67, 58.92, 58.76, 49.54, 43.13, 41.57, 39.07, 38.45, 38.13, 33.13, 32.66, 32.04, 31.41, 29.38, 28.44, 26.41, 24.54. ESMS for $C_{42}H_{73}N_9O_{13}S$ (calcd [M+H]$^+$: 944.5): found [M+H]$^+$ 944.4.

Tetravalent alkyne dendrimer 9

A solution of **8**[24] (206 mg, 0.20 mmol) was stirred in Tesser's base[25] (20 mL) for 3 h. The mixture was acidified with aqueous $KHSO_4$ (1M) to pH 2 and concentrated *in vacuo*. The crude product was taken up in EtOAc (100 mL), and DMF (10 mL) and washed twice with H_2O (50 mL) and brine (50 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated to give the free carboxylic acid (203 mg, quantitative). The acid (203 mg, 0.20 mmol) was dissolved in dry DMF (15 mL) whereupon $^iPr_2EtN$ (0.04 mL, 0.2 mmol) spacer **2** (97 mg, 0.3 mmol) and BOP (134 mg, 0.3 mmol) were added and the mixture was stirred for 3 h. The crude was concentrated *in vacuo* at 60 °C, and pure product was obtained after silica column chromatography (EtOAc–MeOH, 1 : 0–19 : 1) as a yellow oil (192 mg, 72%). ^{1}HNMR (300 MHz, DMSO) δ 8.6 (m, 2H, NH), 8.4 (m, 1H, NH), 8.1 (t, J = 5.5, 4H, NH), 7.0–6.9 (m, 6H, CH_{ar}), 6.7–6.6 (m, 3H, CH_{ar}), 4.1 (m, 4H), 3.9 (m, 8H), 3.6 (m, 4H), 3.5 (m, 8H), 3.3–3.1 (m, 20H), 2.9 (q, J = 6.8, 2H), 2.7 (s, 4H, CH≡C), 2.4–2.2 (m, 16H), 1.7 (t, J = 6.4, 2H), 1.5 (t, J = 6.7, 2H), 1.3 (s, 9H_{Boc}). ^{13}CNMR (75.5 MHz, DMSO)[26] δ (CO) 176.1, 171.3, 171.2, 164.8, 160.8, 142.1, 141.7, (C_{AR})111.5, 109.5, 109.3, (CH≡C) 89.16, (CH_3Boc) 82.8, (CH)76.7, 72.1, 71.6, (CH_2) 45.7, 45.5, 45.2, 44.9, 44.7, 44.4, 44.1, 43.6, 42.7, 41.8, (CH) 39.5, 35.1, 34.7, (CH_2) 33.6, (CH_3) 19.6. ESMS for $C_{68}H_{90}N_8O_{18}$ (calcd. [M+Na]$^+$: 1329.6): found [M+Na]$^+$ 1329.7.

Biotinylated tetravalent galabiose dendrimer 10

A "Click" reaction with galabiose derivative **6**[27] and **9** was performed using the general procedure. The protected tetravalent galabiose dendrimer was isolated by silica gel chromatography (EtOAc–MeOH, 1 : 0–8 : 2) (95.8 mg, 65%). A deprotection reaction was performed by the general procedure. The product was isolated by preparative HPLC (CH_3CN–H_2O), (30 mg, 83%). The product (48 mg, 0.015 mmol) was dissolved in dry DMF (2 mL) under N_2 atmosphere. The solution was basified with DiPEA until pH 8 whereupon succinimidyl-6-(biotinamido)hexanoic acid (10.8 mg, 0.0238 mmol) was added. The mixture was stirred for 72 h and monitored by TLC (EtOAc, MeOH, H_2O 6 : 3 : 1). The crude product was concentrated and purified by HPLC to give the compound **10** (25 mg, 49%). ^{1}H NMR (300 MHz, DMSO): δ = 8.67 (2H, s, NH), 8.40 (1H, s, NH), 8.13 (3H, s, NH), 7.80 (4H, s, $CH_{triazole}$), 7.73 (1H, s, NH), 7.21 (3H, s, CH_{arom}), 7.03 (6H, s, CH_{arom}), 6.66 (4H, s, NH), 6.41 (2H, s, NH), 6.34 (2H, s, NH), 4.81 (d, J = 3.5, 4H, C-1), 4.38–4.26 (m, 16H), 4.11–3.98 (m, 20H), 3.76 (bs, 6H), 3.71–3.54 (m, 20H), 3.47–3.28 (m, 26H), 3.12–3.06 (m,12H), 3.29 (q, J = 6.6, 6H), 2.81–2.77 (m, 10H), 2.70 (t, 4H), 1.99 (q, J = 6.6, 14H), 1.89 (s, 2H), 1.46–1.32 (m, 20H), 1.25–1.19 (m, 18H). ^{13}C NMR (from HSQC, DMSO): δ = 122.6, 106.4, 104.8, 104.1, 101.2, 77.73, 74.91, 71.86, 70.21, 69.5, 69.03, 67.15, 66.45, 65.51, 61.51, 60.81, 59.63, 55.64, 46.7, 40.83, 40.12, 39.89, 38.95, 38.48, 35.19, 34.95, 30.02, 29.08, 28.14, 26.26, 25.32, 25.08, 21.32, 18.03, 16.38, 12.62. MALDI-TOF for $C_{139}H_{215}N_{23}O_{63}S$ (calcd. $[M+Na]^+$: 3269.4): found $[M+Na]^+$ 3269.1.

Coating of magnetic submicron-particles with biotinylated dendrimers 4, 5 and 10

Magnetic submicrometer particles (sold as nanoparticles) (10 mg ml^{-1}, Streptavidin beads, Ø 250 nm, 1.1 × 10^{14} pieces/g, 1.25 g cm^{-3}, Chemicell, Germany) were washed three times with phosphate buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 0.8 mM Na_2HPO_4, 1 mM KH_2PO_4). Biotinylated ligand/dendrimer were mixed with the particles in the ratio 1 nmol of ligand/dendrimer per mg of the particles at room temperature for 30 min. After the coating, the particles were washed three times with PBS.

Coating of magnetic microparticles with the biotinylated galabiose dendrimers

Magnetic microparticles (MagSelect SA, Sigma, USA) were washed three times with PBS. The microparticles were incubated with mono- or tetravalent galabiose in the ratio 2 nmol of biotinylated ligand/dendrimer per µL of the microparticles at room temperature for 30 min. After the coating, the particles were washed three times with PBS.

Bacterial detection experiments

The galabiose-binding *S. suis* strain D282 was cultivated in Todd-Hewitt broth supplemented with 5% (w/v) yeast extract at 37 °C. The optical density (OD_{600}) of the overnight grown bacteria was adjusted using Todd-Hewitt broth to make various dilutions. 500 µL of a bacterial dilution and 25 µL of the coated glycoparticles were incubated at room temperature for an hour. Two parallel samples of each bacterial dilution were run in each assay. After the incubation, the particles were washed once with PBS, suspended in 100 µL of PBS and transferred into a 96-well plate. To detect the live bacterial cells, 100 µL of BacTiter-Glo reagent (Promega, Madison WI, USA) was added, the cells were mixed for 5 min and the luminescence in each well was measured with Victor² multilabel counter (PerkinElmer, Turku, Finland).

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