Biotinylated phosphorus dendrimers as control line in nucleic acid lateral flow tests

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Lateral flow assays (LFA) are affordable, easy-to-use, qualitative rapid test for clinical diagnosis in non-laboratory environments and low-resource facilities. The control line of these tests is very important to provide a valid result, confirming that the platform operates correctly. A clear, non-diffused line is desirable. The number of coloured nanoparticles that reach the control line in a

positive test can be very small and they should all be trapped efficiently by the molecules adsorbed there. In this work, we proposed the use of robust biotinylated dendrimers of two different generations as signal amplifiers in control lines of LFA, able to react with streptavidin modified gold nanoparticles. Beside the synthesis and characterization, the analytical performance as control lines will be studied, and their response will be compared with other commercially available biotinylated molecules. Finally, the utility of the dendrimer implemented in a NALF (Nucleic Acid Lateral Flow) strip was also demonstrated for the detection of the amplicons obtained by double-tagging PCR (polymerase chain reaction) for the detection of *E. coli* as a model of foodborne pathogen.

INTRODUCTION

Dendrimers are characterized by their monodisperse three-dimensional hyperbranched structures and by their multiple terminal functions that are generally accessible and easy to modify.[1] Taking advantage of the large number of external moieties that increases in higher generations, dendrimers have been used in sensors for the recognition of different targets, including metal cations (ex. Co^{2+}, Pd^{2+}, Cu^{2+}, Ba^{2+}),[2,3] small anions ($H_2PO_4^-$, ATP^{2-}),[4] toxic gases (SO_2),[5] explosives (TNT),[6] oxidants (H_2O_2),[7] volatile organic compounds,[8] among many others.[1] Their use in biosensors has been also largely developed for the recognition of biomolecules (glucose, avidin, dopamine, thrombin, etc.) and cell components.[1,9] Among these biosensors, DNA microarrays have received considerable attention,[10] being phosphorus dendrimers (PPH) one of the most studied structures. However, for other applications, poly(amidoamine) (PAMAM) dendrimers are the most used.[1,11,12,13] In these applications, dendrimers are implemented as signal amplifiers upon reaction

with the target, increasing thus the sensitivity of the device. The most common readout approaches are based on fluorescence, UV-vis-absorption or luminescence emission, electrochemical signal, surface plasmon resonance, surface enhanced Raman scattering, and mass by using quartz crystal microbalance.[1] It has been reported that dendrimer-based platforms also allow not only an improvement in target capturing ability, but also in sensitivity, specificity, reliability and stability of sensing and biosensing devices.

However, the accurate identification of patients requiring treatment in low resource settings still remains a major stumbling block to disease control due to the lack, in some instances, of rapid, cost effective diagnostic tests that can be handled for unskilled personnel.[14] In this direction, the FDA defines the characteristics that a diagnostic test should ideally have. Low complexity for a test includes the final-user interpretation and level of training required, the number of manual manipulations and intervention steps, and the instrumentation requirements.[15] The preeminent formats under development as rapid diagnostic tests (RDTs) are lateral-flow. The lateral-flow assay (LFA) introduced in 1988 by Unipath, is the most common commercially available point of care diagnostic format including the pregnancy test.[16] It consists mainly in a porous membrane able to transport spontaneously fluids containing the analytes. Upon addition of the sample, the analyte interacts with the visible signal-generating system (generally with antibodies tagged with colloidal gold, latex spheres or dyed polystyrene). Then, this complex migrates along the strip up to the test line and interacts with the deposited antibody therein. The affinity reaction occurs while a visible signal is generated. Excess of tagged antibodies is finally detected in the control line, to confirm that the device is working properly. Although many methodological advances and analytical simplification have been done in the field, sensitivity should be improved. Positive charged PAMAM dendrimers have been used to generate gold nanoparticle[17,18] and magnetic

particle[19] aggregates, and nanochains[20] that have been used as colored tagger to increase sensitivity in test lines of LFA, compared to individual nanoparticles. Covalent conjugation of latex particles with antibodies and coumarin-derived dendrimers were the base of a smartphone-based fluorescent diagnostic lateral flow immunoassay for H5N1 viruses.[21] In the four examples, dendrimers are used in the conjugate pad, interacting with gold nanoparticles. To the best of our knowledge, no example of dendrimers used as control line reactants has been described up to now. Taking into account their high number of surface functions, they could interact easily and efficiently with residual commonly used gold nanoparticles.[22] Most frequently used proteins[23] and antibodies[24] could be replaced by home-made, cheaper and more robust macromolecules.

Avidin and streptavidin bind four moles of biotin per mole of protein with an extraordinary affinity ($Ka=10^{15}\ M^{-1}$).[25,26,27,28] Some applications in which the strept(avidin)-biotin interaction have been used include ELISA, immunohistochemical staining, Western, Northern and Southern blotting, immunoprecipitation, cell-surface labelling, affinity purification, fluorescence-activated cell sorting (FACS), among many others. The valeric acid side chain of the biotin molecule can be derivatised to incorporate reactive groups that are used to attach biotin to biomolecules, including peptides, antibodies, enzymes, receptors, nucleic acids, and lately dendrimers without significantly altering their biological activity.[28]

Biotinylated PAMAM dendrimers have been used as: drug delivery nanocarriers for cancer therapy and diagnosis,[29,30,31,32,33] versatile platform for other biomedical applications,[34] DNA chips,[35] contrast agents for MR imaging,[36] and important components in electrochemiluminescence biosensors.[37] Other biotinylated dendrimers are rare, only two examples of (L-glutamic acid) and poly(lysine) internal structures have been used as drug carriers.[38,39] An example of special biotinylated DNA dendrimers has been patented as part of the

conjugate path in lateral flow assays to amplify the desired signal;[40] playing similar role as previously reported positive charged PAMAM dendrimers.[17,20] Phosphorus dendrimers (PPH)[41] are robust structures also used for sensor applications.[42,43] A micromechanical biosensor based in DNA-PPH dendrimers hybridized with biotinylated oligonucleotides has been reported, with biomolecular recognition based on their interaction with streptavidin-conjugated gold nanoparticles.[44] It should be highlighted that the solubility of PPH dendrimers can be modulated by modification of their chemical composition and size.[45]

In this work we propose the use of two different generations of biotinylated phosphorus dendrimers as components of the control line of LFA for the detection of streptavidin-tagged gold nanoparticles. Signal amplification of different size biotinylated macromolecules at different concentrations, their performance over time, and the comparison of their analytical performance with the most commonly used biotinylated species will be also discussed. Finally, the utility of the dendrimer implemented in a NALF (Nucleic Acid Lateral Flow) strip was also demonstrated for the detection of the amplicons obtained by double-tagging PCR (polymerase chain reaction) for the detection of *E. coli* as a model.

EXPERIMENTAL SECTION

Materials and methods. All commercially acquired reagents were used as received. When required solvents were dried used standard procedures. Reactions requiring inert atmosphere were conducted under nitrogen using standard Schlenk techniques. All other reactions were performed employing standard organic synthesis protocols. Thin layer chromatography (TLC) was performed using Merck aluminium backed plates of TLC Silica gel 60 F254; the plates were revealed using UV light. Standard Flash Column chromatography was accomplished using silica gel (60 Å pore

size, 230-400 μm mesh size). Molecular sieves were from Sigma-Aldrich 8-12 mesh, 4 Å pore size.

Amine-PEG_3-biotin was purchased from Thermo Scientific. InnovaCoat® GOLD 40 nm Streptavidin gold nanoparticles (streptAv-AuNPs) were purchased from Expedeon (Cambridge, UK). The concentration of the streptAv-AuNPs was 10 OD (surface plasmon resonance absorption peak at 530 nm; molar extinction 8.42 10^9 M^{-1} cm^{-1}, molar concentration 1.18 10^{-9} mol L^{-1}). Anti-digoxigenin (anti-DIG, Ref 11214667001) was purchased from Roche Diagnostics, while biotin (Ref B4501) and albumin, biotin labeled bovine (BSA-biotin, Ref A8549) were from Sigma-Aldrich. The biotinylated antibody (IgG-biotin, Ref ab69255), was purchased in Abcam.

The buffer solutions were prepared with milliQ water and all other reagents were in analytical reagent grade (supplied from Sigma and Merck). The composition of these solutions are: a) conjugate diluting buffer (2 mmol L^{-1} borate pH 7, 10 % w/v sucrose); b) sample pad buffer (0.01 mol L^{-1} phosphate buffer pH 7.4, 1% BSA, 0.05 % Tween 20); c) running buffer (0.01 mol L^{-1} phosphate buffer pH 7.4, 1% BSA, 0.05 % Tween 20).

Spectra were recorded using Bruker spectrometers DXP-360 and AVANCE-III 400 (360 MHz (^{1}H); 90 MHz (^{13}C) and 400 MHz (^{1}H); 101 MHz (^{13}C); 162 MHz (^{31}P) respectively). ^{1}H and ^{13}C chemical shifts are reported in ppm relative to tetramethylsilane, using residual proton and ^{13}C resonances from solvent as internal standards. ^{31}P chemical shifts are reported relative to H_3PO_4 85% in water. Infrared spectra were recorded using a Bruker Tensor 27 instrument equipped with an ATR Golden Gate cell and a diamond window. MALDI-TOF MS were performed in a Bruker BIFLEX with Reflectus Modus. Elemental analyses were done by the *Serveis d'Anàlisi Química* of the *Universitat de Autònoma de Barcelona*. Elemental analyses of C, H and N were performed

using an elemental analyser EA-1108 CE Instrument of Thermo Fisher Scientific with BBOT as an internal standard. Atomic force microscopy (AFM) was recorded on an Agilent 5500 AFM/SPM Microscope. AFM image process and render were done with WSxM software (Nanotech Electronica S. L. Spain). Transmission electron microscopy has been performed in a HITACHI H-7000 (100kV). Program ImageJ (Fiji) has been used to measure the diameter of particles.

Glass fiber conjugate pad (GFCP083000) and cellulose fiber sample pad strips (CFSP203000) were purchased from Millipore. Adhesive Backing Cards were obtained from Kenosha C.V. (Netherlands) and nitrocellulose membranes (FP120HP) as well as the absorbent pads (CF7) were purchased from GE Healthcare Europe. Lateral Flow Reagent Dispenser from Gentaur combined with the KDS Legato™ 200 series siringe pump from KD Scientific Inc. (Holliston, MA) was used to dispense the test and control line. Software ImageJ was used to quantify the intensity of color in strips.

Synthesis of organic molecules

Compound **1**[46] and dendrimers **G1** and **G4**, containing 12 and 96 aldehyde groups on the surface respectively, were prepared following previously described procedures.[47]

*Synthesis of compound **1***.[46] A round-bottom flask equipped with a $CaCl_2$ tube and magnetic stir bar was charged with the phosphorodichloridothioic hydrazide, methyl(phenylmethylene) (200 mg, 0.75 mmol, 1 equiv), 4 hydroxybenzaldehyde (183 mg, 1.5 mmol, 2 equiv) followed by the addition of dry THF (0.1 M). Next, dry cesium carbonate (733 mg, 2.25 mmol, 3 equiv) was added in one portion. The reaction mixture was stirred at room temperature for 12 h. Upon completion (checked by ^{31}P NMR) the reaction mixture was filtered and concentrated in vacuo. The residue was washed with dry pentane, then was dissolved in a minimal amount of chloroform, an excess

of pentane was added, and a brown solid was appeared and discarded. The liquid layer was concentrated and the purification was accomplished via flash column chromatography over silica gel (dichloromethane:hexane 1:9) afforded the desired product (175 mg, 54% yield) as a colorless oil. ^{1}H NMR (360 MHz, $CDCl_3$): δ 9.95 (s, 2H), 7.87 (dt, *J* = 8.8 Hz, *J* = 1.0 Hz, 4H), 7.70 (m, 2H), 7.60 (d, *J* = 1.8 Hz, 1H), 7.30-7.40 (m, 7H), 3.41 (dd, *J* = 10.9 Hz, *J* = 1.0 Hz, 3H) ppm. ^{13}C NMR (90 MHz, $CDCl_3$): δ 190.9, 155.3, 140.9 (d, *J* = 14.1 Hz), 134.4, 133.7 (d, *J* = 2.0 Hz), 131.5 (d, *J* = 2.0 Hz), 129.9, 128.9, 127.1, 122.1 (d, *J* = 4.9 Hz), 33.0 (d, *J* = 13.9 Hz) ppm. ^{31}P NMR (162 MHz, $CDCl_3$): δ 60.4 ppm. IR (ATR): ν_{max} 3066, 3028, 2825, 2737, 1697, 1595, 1499, 1190, 1151, 905 cm^{-1}. HR-MS (MALDI-TOF): m/z calcd for $C_{22}H_{19}N_2O_4PS$ ($[M+Na]^+$): 461.0701, found: 461.0695 [$M+Na^+$].

*Synthesis of compound **3**.* A round-bottom flask was charged under inert atmosphere with **1** (8 mg, 0.02 mmol, 1 equiv),[46] NH_2-PEG_3-biotine **2** (19 mg, 0.045 mmol, 2.2 equiv) followed by the addition of DMF-d_7 (0.7 mL, 0.028 M), and 4 Å molecular sieves. The reaction mixture was stirred at room temperature for 4 days. When the aldehyde was consumed (monitored by ^{1}H NMR), the imine reduction was *in situ* carried out. To achieve this transformation, BH_3SMe_2 in CH_2Cl_2 (35μL, 0.2 mmol, 10 equiv) was added over the previous reaction solution, and the resulting mixture was stirred at room temperature for 36 h (until ^{1}H NMR imine signal was disappeared). The crude was quenched by addition of methanol (0.3 mL), concentrated under reduced pressure and lyophilized. The desired product (22 mg, 89% yield) was obtained without further purification as a colorless oil. ^{1}H NMR (400 MHz, DMF-d_7): δ 8.07 – 7.96 (m, 2H), 7.84 – 7.74 (m, 2H), 7.51 – 7.33 (m, 6H), 7.28 – 7.19 (m, 3H), 6.20 (d, *J* = 17.4 Hz, 2H), 4.46 (t, *J* = 6.3 Hz, 2H), 4.35 – 4.23 (m, 2H), 3.66 – 3.51 (m, 8H), 3.52 – 3.44 (m, 11H), 3.45 – 3.27 (m, 20H), 3.25 – 3.15 (m, 3H), 2.64 – 2.52 (m, 4H), 2.23 – 2.14 (m, 4H), 1.77 (ddt, *J* = 12.5, 9.4, 6.2 Hz, 2H), 1.69 – 1.51 (m, 6H), 1.51 –

1.36 (m, 4H) ppm. ^{13}C NMR (101 MHz, DMF-d_7): δ 173.5 , 164.0 , 150.8 (d, J = 7.4 Hz), 142.3 (d, J = 13.9 Hz), 138.0 , 136.5 , 131.0 , 130.5 , 130.4 , 129.8 , 128.0 , 121.9 (d, J = 4.9 Hz), 121.8 , 71.4 , 71.3 , 71.2 , 71.1 , 70.7 , 70.5 , 62.7 , 62.5 , 61.1 , 41.2 , 40.0 , 36.6 , 33.8 (d, J = 12.1 Hz), 29.6 (d, J = 9.0 Hz), 26.7 ppm. ^{31}P NMR (162 MHz, Acetone-d_6): δ 63.9 ppm. IR (ATR): ν_{max} 3272, 2865, 1693, 1636, 1551, 1503, 1450, 1399, 1261, 1191, 1123, 960, 920, 778, 697 cm^{-1}. Elemental analysis for $C_{58}H_{87}N_{10}O_{12}PS_3$ calculated C 56.20, H 7.05, N 11.26; found: C 56.31, H 6.84, N 11.45.

Synthesis of compound ***G1-biotin***. A round-bottom flask was charged under inert atmosphere with dendrimer **G1** (10 mg, $3.5 \cdot 10^{-3}$ mmol, 1 equiv),[47] NH_2-PEG_3-biotine **2** (18 mg, 0.042 mmol, 12 equiv) followed by the addition of DMF-d_7 (0.6 mL, 6.7 mM), and 4 Å molecular sieves. The reaction mixture was stirred at room temperature for 4 days. When the aldehyde was consumed (monitored by ^{1}H NMR), the imine reduction was *in situ* carried out. To achieve this transformation, BH_3SMe_2 in CH_2Cl_2 (40 µL, 0.42 mmol, 120 equiv) was added over the previous reaction solution, and the resulting mixture was stirred at room temperature for 3 days (until ^{1}H NMR imine signal is disappeared). The crude was quenched by addition of methanol (1 mL), concentrated under reduced pressure, and lyophilized. The desired product (18 mg, 66% yield) was obtained without further purification as a white solid. ^{1}H NMR (400 MHz, DMF-d_7): δ 7.91 – 7.71 (m, 24H), 7.58 (d, J = 8.1 Hz, 6H), 7.46 – 7.11 (m, 48H), 6.44 (d, J = 33.6 Hz, 12H), 4.46 (t, J = 6.2 Hz, 12H), 4.28 (t, J = 6.2 Hz, 12H), 3.87 – 3.71 (m, 48H), 3.67 – 3.42 (m, 168H), 3.35 – 3.26 (m, 18H), 3.19 (s, 12H), 2.74 – 2.64 (m, 24H), 2.18 (t, J = 8.0 Hz, 24H), 1.77 – 1.68 (m, 12H), 1.67 – 1.49 (m, 36H), 1.47 – 1.33 (m, 24H) ppm. ^{13}C NMR (101 MHz, DMF-d_7): δ 173.6, 164.2, 163.1, 157.7, 157.3, 156.8, 152.3, 152.2, 152.1, 151.6, 151.6, 151.5, 138.2, 135.3, 133.4, 133.0, 131.3, 130.8, 130.5, 129.6, 129.0, 124.3, 122.2, 122.1, 121.9, 121.6, 119.4, 71.4, 71.3, 71.2,

71.2, 71.1, 71.1, 70.7, 70.4, 67.4, 64.0, 62.7, 61.03, 59.4, 56.9, 56.9, 53.3, 53.3, 41.2, 41.2, 40.0, 32.8, 29.6, 29.5, 28.0, 28.0, 26.7, 26.6, 23.5, 20.2, 14.7 ppm. ^{31}P NMR (162 MHz, DMF-d_7): δ 62.8, 8.4 ppm. IR (ATR): ν_{max} 3288, 2921, 2866, 1686, 1649, 1552, 1505, 1459, 1195, 1162, 1138, 1098, 945, 785, 632 cm^{-1}. Elemental analysis for $C_{348}H_{516}N_{63}O_{78}P_9S_{18}$ calculated C 54.38, H 6.77, N 11.48; found: C 54.49, H 6.95, N 11.68.

Synthesis of compound ***G4-biotin***. A round-bottom flask was charged under inert atmosphere with dendrimer **G4** (10 mg, $3.25 \cdot 10^{-4}$ mmol, 1 equiv),[47] NH_2-PEG3-biotine **2** (13 mg, 0.03 mmol, 96 equiv) followed by the addition of DMF-d_7 (0.6 mL, 6.7 mM), and 4 Å molecular sieves. The reaction mixture was stirred at room temperature for 5 days. When the aldehyde was consumed (monitored by ^{1}H NMR), the imine reduction was *in situ* carried out. To achieve this transformation, $BH_3SMe_2 \cdot CH_2Cl_2$ (31 µL, 0.33 mmol, 1015 equiv) was added over the previous reaction solution and the resulting solution was stirred at room temperature for 3 days (until imine signal is disappeared). The crude was quenched by addition of methanol (1 mL), concentrated under reduced pressure, and lyophilized. The desired product (18 mg, 82% yield) was obtained without further purification as a yellow oil. ^{1}H NMR (400 MHz, DMF-d_7): δ 7.87 (s, 336H), 7.57 (s, 90H), 7.33 (d, J = 42.6 Hz, 408H), 6.41 (t, J = 37.5 Hz, 180H), 5.75 (s, 40H), 4.46 (s, 96H), 4.29 (s, 96H), 4.17 – 3.07 (m, 1998H), 3.05 – 2.54 (m, 96H), 2.46 (s, 192H), 2.17 (s, 192H), 1.83 – 1.11 (m, 576H). ^{13}C NMR (101 MHz, DMF-d_7): δ 173.7, 173.0, 164.1, 163.1, 133.0, 130.6, 129.7, 129.5, 129.5, 122.9, 122.7, 122.0, 71.2, 71.1, 70.6, 68.7, 68.3, 67.4, 62.6, 61.0, 56.9, 41.3, 41.1, 41.0, 40.0, 40.0, 36.5, 32.1, 31.3, 31.2, 29.6, 29.4, 27.0, 26.4 ppm. ^{31}P NMR (162 MHz, DMF-d_7): δ 62.51, 7.48 ppm. IR (ATR): ν_{max} 3292, 2922, 2866, 1639, 1552, 1503, 1452, 1387, 1192, 1096, 962, 921, 841, 783, 637 cm^{-1}. Elemental analysis for $C_{3120}H_{4464}N_{567}O_{666}P_{93}S_{186}$ calculated C 53.98, H 6.49, N 11.44; found: C 54.15, H 6.63, N 11.71.

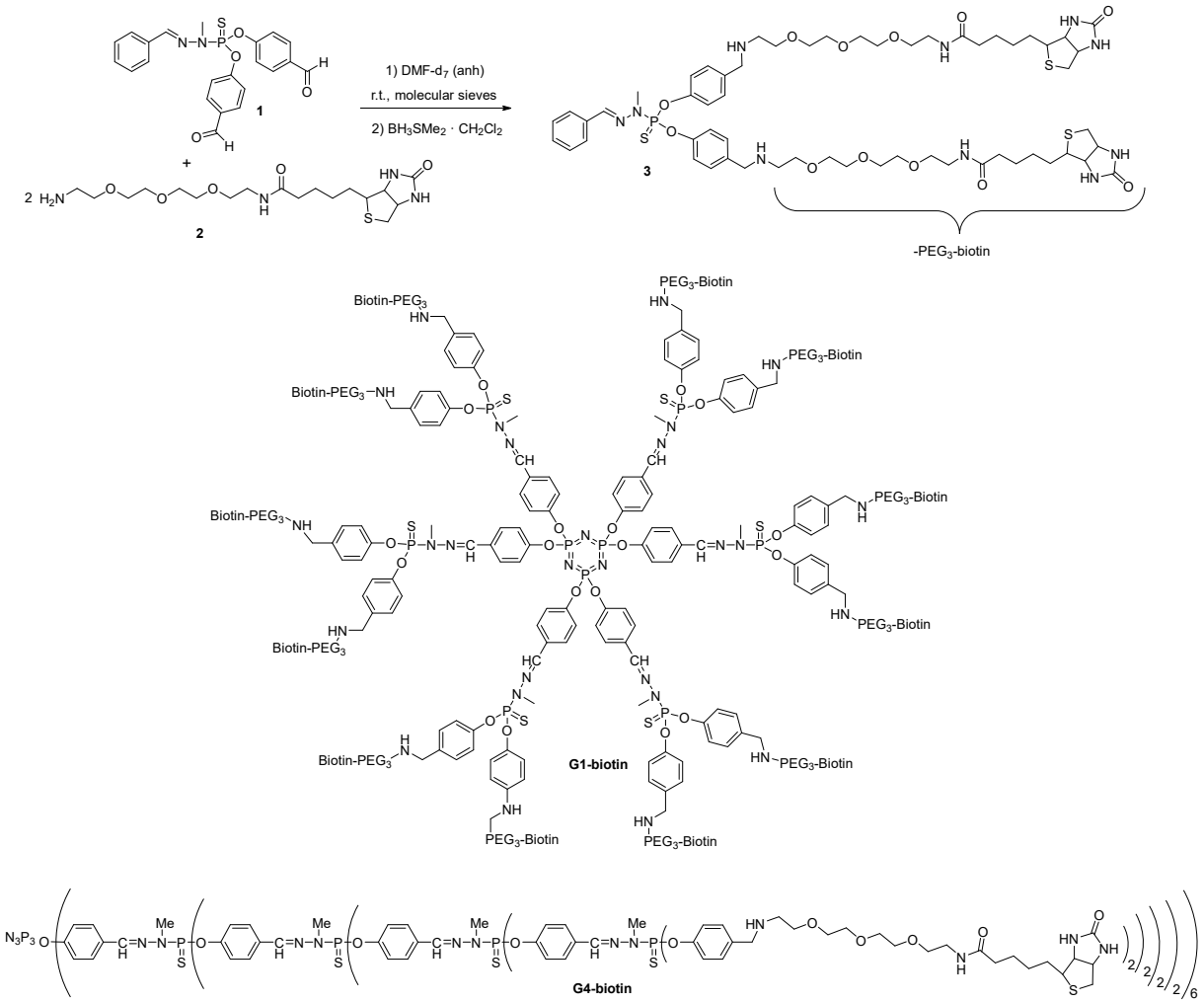
Preparation of lateral flow strips. Commercial streptAv-Au NPs were diluted 20 times in conjugate diluting buffer and embedded in the conjugate path of the nitrocellulose strips with the dispenser and dried for 2 h. The antibodies and the positive control biotinylated reporter were also dispensed on the nitrocellulose membrane and were then dried at RT for 1h. For Bacterial strain growth conditions and DNA extraction see previous reported results.[22] For more detailed data see SI.

RESULTS AND DISCUSSION

First of all, we decided to prepare three phosphorus derived molecules containing 2, 12 and 96 biotin moieties in their structures (compounds **3**, **G1-biotin**, and **G4-biotin** respectively, Scheme 1). Compound **3** was prepared as a simple model molecule containing the main chemical structure of phosphorus dendrimers, in order to compare the existence or not of a dendrimer effect in their application as control line amplifiers in LFA. Compound **1** was prepared from a reaction of a previously described phosphonothioic dichloride derivative[46] with *p*-hydroxybenzaldehyde in basic media at room temperature (see SI). Commercially available amine-PEG_3-Biotin ((+)-biotinyl-3,6,9-trioxaundecanediamine), **2**, was used to incorporate biotin moieties on surface of phosphorus molecules. This derivative containing in its structure a short poly(oxyethylenated) chain was selected to provide better water solubility of final molecules. Compound **1**, and two phosphorus dendrimers of the first and fourth generation (**G1** and **G4**), created from cyclotriphosphazene core,[47] containing aldehyde groups on the surface, reacted with 2, 12, and 96 equivalents respectively of biotin derived compound **2**, in deuterated DMF, in the presence of molecular sieves. The formation of the imine derivatives was followed by the evolution of the signals in the ^{1}H NMR spectra; aldehyde groups (9.90-10.10 ppm) disappeared at the same time

that the signals of the proton of imine groups appeared (8.30-8.40 ppm). This process lasted 4 to 5 days. Then a big excess of $BH_3 \cdot SMe_2$ was added, and after three days the reduction of imine groups was accomplished, as observed by the disappearance of the corresponding proton signal. The excess of borane was removed by quenching the reaction with an excess of MeOH. The absence of signal in ^{10}B NMR confirmed its complete elimination. ^{1}H-NMR spectra of both dendrimers showed large signals, probably due to the high mobility of external functional groups and the generation of intra and intermolecular hydrogen bonds. Reactions were quantitative, but after sample extraction/recuperation processes for monitoring the evolution of the reactions (two consecutive steps) by NMR experiments, and final evaporation, the total isolated yields were 89 % for **3**, 66 % for **G1-biotin**, and 82% for **G4-biotin**, without isolation of imine derivatives.

The size of **G4-biotin** dendrimer was studied when deposited on a surface. When dendrimer **G4-biotin** was deposited on mica using MeOH as solvent and studied by atomic force microscopy (AFM), aggregates around 590 nm were observed. When a highly diluted solution of dendrimer **G4-biotin** in water/DMF (9:1) was studied by transmission electron microscopy (TEM), using uranyl acetate as negative stain, small nanoparticles of an average of 8.7 ± 1.2 nm of diameter were observed (419 particles were measured, see SI). This value is slightly higher than other previously reported for PPH dendrimers of the same generation with other surface functions.[48] Taking into account the diameter distribution histogram, particles from 6.5 to 12.8 nm were observed, so probably aggregates of two units could be formed in the preparation of the sample. So, during deposition process aggregates of different sizes could be formed, depending the experimental conditions used.



Scheme 1. Synthesis of biotinylated molecules **3**, **G1-biotin** and **G4-biotin**

We prepared diluted solutions 1 mg mL^{-1} of compounds **3**, **G1-biotin**, and **G4-biotin** in water/DMF (9:1). Some portion of an organic solvent was required to solubilize the internal structure of dendrimers, part that is hydrophobic.[45] These solutions were dispensed on the nitrocellulose membrane and were then dried at room temperature for 1h. Then, the running buffer was added on the sample pad of the strip containing the streptavidin-modified gold nanoparticles (as detailed described in SI). When the buffer solution was added to the sample pad and diffuses through the strip, the streptAv-AuNPs moved also along the strip and were captured by the strong

affinity interaction (Ka=10^{15} M^{-1})[28] among the streptavidin on the AuNPs and the biotin linked to the phosphorus dendrimers previously adsorbed on the strip. A clear red line should be observed as a positive reaction (as shown in Figure 1, design of strips).

First of all, a comparative study of the analytical performance of compound **3** and dendrimers **G1-** and **G4-biotin** was performed compared to free biotin as well as other commercial biotinylated biomolecules, including BSA (**BSA-biotin**) and biotinylated antibody (**IgG-biotin**),[49,50] and the results are shown in Figure 1, down panel. It is important to highlight that the immobilization of the molecules on nitrocellulose is due to physical forces, which considered individually, are weak. However, large molecules as dendrimers and proteins (including BSA and antibodies) can establish large number of interactions. Accordingly, free biotin molecule gave no signal, as well as compound **3**, probably due to desorption upon the addition of the running buffer (1.560·μmol mL^{-1} dispensed). However, the solubility of modified dendrimers **G1-biotin** and **G4-biotin** in water was very low, and after being deposited on the strip, they did not move when the solution passed over, and a clear visual readout was thus achieved, showing that both molecules were good as control line reporters.

As shown in Figure 1, in all instances in which macromolecules were used, clear visual red lines were formed on the nitrocellulose membrane of the strip due to the accumulation of functionalized gold nanoparticles in the control line at a concentration of 5.90 10^{-11} mol L^{-1} (0.5 OD), as recommended by the manufactured for this application. It should be highlighted that thinner and non-diffuse lines were obtained when dendrimers were used. This result confirmed the need of high molecular weight, low water soluble, biotinylated macromolecules to form the control line. These dendrimers not only showed the proper solubility, but also they contained, for similar w/v concentrations (1.00 mg mL^{-1}), higher number of biotin moieties, giving better performance (1.38,

1.56, and 0.17 μmol biotin mL^{-1} for **G4-biotin**, **G1-biotin**, and **BSA-biotin**, respectively). This information is not provided from commercial source for the **IgG antibody**. The performance of the control line is thus not only dependant on the number of biotin moieties, but also on the nature of the macromolecule. It is important to highlight that the cost of the biological reagents is much higher than the biotinylated dendrimers, and a higher stability for the dendrimers can be also expected compared to the proteins (BSA or antibody).

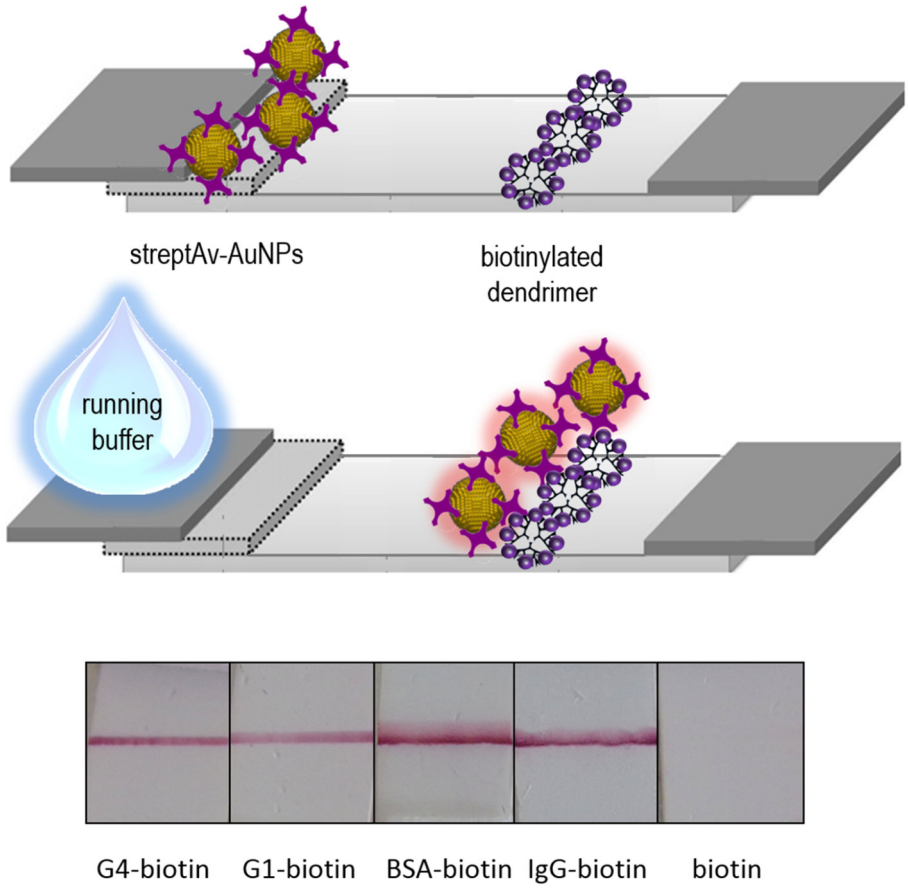


Figure 1. Up: General scheme of design of strips, showing the details of the interaction between the biotinylated biomolecule (in this instance, the dendrimer) and the streptavidin-modified gold nanoparticles (streptAv-AuNPs) used as signal generating system. Down: Images of strips of

LFA tests showing interaction between different biotinylated macromolecules and free biotin with streptAv-AuNPs at 5.90 10^{-11} mol L^{-1} (0.5 OD). Concentrations of dispensed solutions: 1.00 mg mL^{-1} for **G1-biotin**, **G4-biotin**, **BSA-biotin**, and **IgG-biotin** and 0.38 mg mL^{-1} for biotin. Further results (including replicates) are provided in SI.

In order to compare the performance of dendrimers, further characterization was done at different concentrations of the dispensed dendrimers, ranging from 0.001 to 1.000 mg mL^{-1}. (as described in detail in SI). The comparative results are shown in Figure 2.

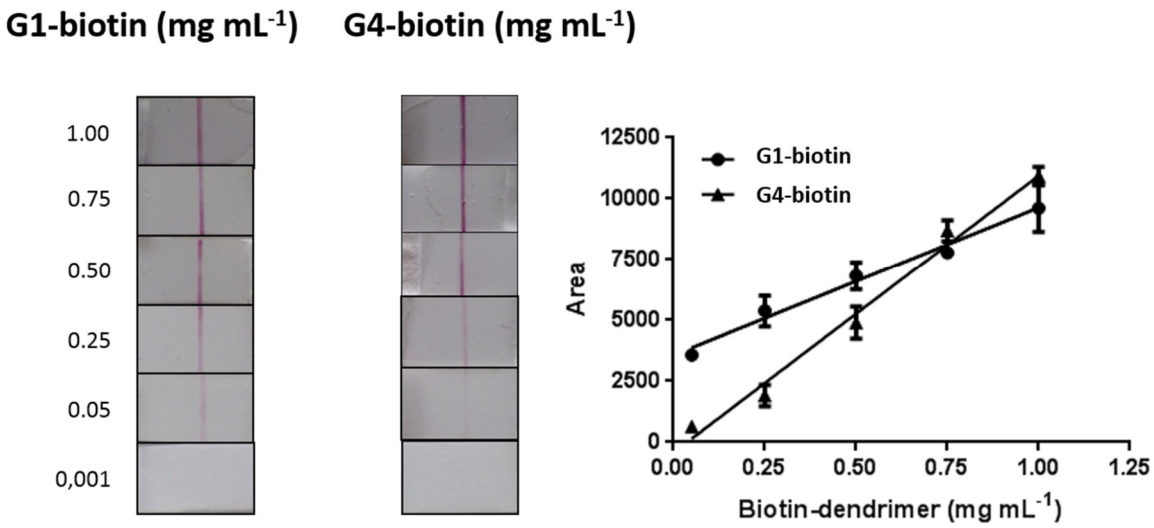


Figure 2. LFA images using different concentration of dispensed dendrimers solutions (9:1 water:DMF) as control line, after interaction with 5.90 10^{-11} mol L^{-1} (0.5 OD) streptAV-AuNPs. The comparative calibration plot of the dendrimers **G1-** and **G4-biotin** are also shown. Further results (including replicates) are provided in SI.

As shown in Figure 2, at lower concentration of dendrimers (0.25 and 0.05 mg mL-1) **G1-biotin** showed clearly more intense bands compared to **G4-biotin**. However at higher concentration (1 mg mL^{-1}) the intensity given by both dendrimers is quite similar, probably due to the saturation of the streptavidin binding sites of the our streptAv-AuNPs providing the same visual signal. Moreover, at this concentration level, and accordingly to the results, clear well defined intense red control lines were obtained. For that reason, 1 mg mL^{-1} will be considered for further experiments. Importantly, diffusion of the line was not observed in any case. The results of the tests could be also estimated by measuring the intensity of the red bands with the software ImageJ (as detailed explained in SI). The relative areas obtained by processing the images were fitted using a linear regression (GraphPad Prism Software) (R^2 = 0.9390 and 0.9770 for **G1-** and **G4-biotin** respectively).

With this experiment, it was confirmed that a higher sensitivity of the biotinylated first generation dendrimer (**G1-biotin**) at lower concentration (0.05 mg mL^{-1}) was achieved. However, at higher concentration (> 0.5 mg mL^{-1}) both dendrimers show similar response and can be interchangeably used. Despite this, more expensive and difficult to prepare higher generation dendrimers are not required for this application.

Due to the configuration of a lateral flow strip, it is important to highlight that the signal generating system, in this instance streptAv-AuNPs, interacts firstly with the test line, and after that with the control line (as schematically shown in SI, *vide infra* Figure 5). As a consequence, it is expected that in the case of a negative result, all the streptAv-AuNPs will arrive to the control line. On the contrary, in case of a positive results, (and depending on the concentration of the analyte in the sample), some of the streptAv-AuNPs will interact on the test line and the remaining streptAv-AuNPs will arrive to the control line. It is thus extremely important to determine the minimum

amount of streptAv-AuNPs which is able to provide a positive control line and, as such, a valid test. The results shown in Figure 3 represent the comparative calibration plots for **G1-** and **G4-biotin** dendrimer (1 mg mL^{-1}) upon increasing concentration of streptAv-AuNPs.

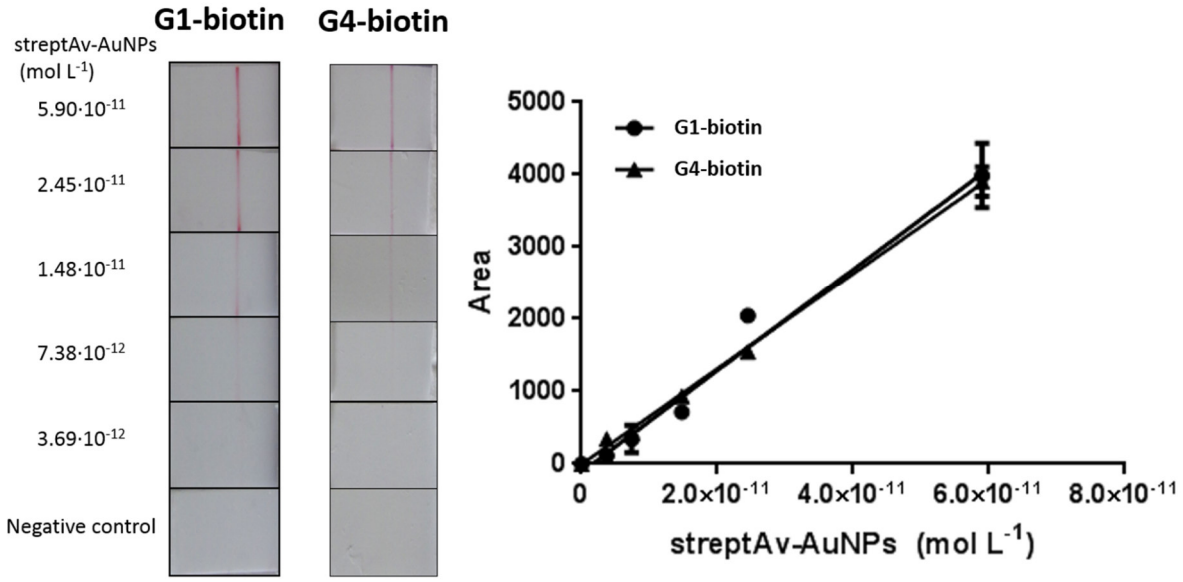


Figure 3. LFA images using different concentration of signal generating system (streptAv-AuNPs) in the conjugation pad and 1 mg mL^{-1} of dispensed dendrimers solutions (9:1, water:DMF) as control line. The comparative calibration plot of the dendrimers **G1-** and **G4-biotin** are also shown. Further results (including replicates) are provided in SI.

As shown in Figure 3, both dendrimers showed similar sensitivity (similar slope-value). Regarding the limits of detection (LODs), both dendrimers are able to clearly distinguish with the naked eye as low as 7.38 10^{-12} mol L^{-1} streptAv-AuNPs. However, lower concentration 3.69 10^{-12} mol L^{-1} streptAv-AuNPs can be distinguish by measuring the intensity of the red bands with the software ImageJ. The relative areas obtained by processing the images were fitted using a linear regression (GraphPad Prism Software) (R^2 = 0.9766 and 0.9960 for **G1-** and **G4-biotin** respectively).

The stability was studied for a period of time of 1 month. This experiment was performed by preparing 5 strips per each of the dendrimers (**G1-** and **G4-biotin**) at a concentration of 1 mg mL^{-1} (as well as their corresponding replicates). The strips were kept at RT for 1 month and the tests were performed on a weekly basis. The results are shown in Figure 4. Outstanding stability was achieved with both types of dendrimers.

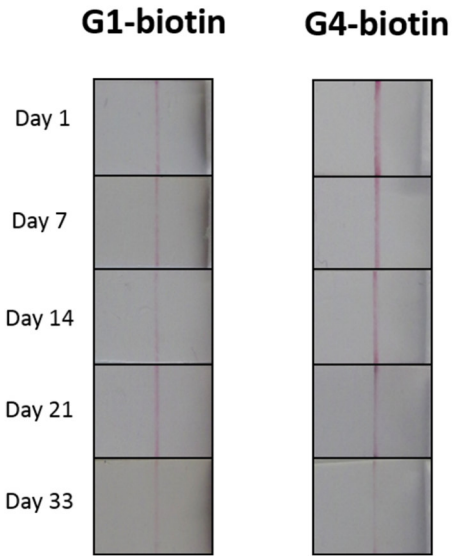


Figure 4. Stability study at a 1 mg mL^{-1} of dispensed **G1-** and **G4-biotin** dendrimers solutions (9:1, water:DMF) as control line and a concentration of signal generating system (streptAv-AuNPs) in the conjugation pad of 2.45 10^{-11} mol L^{-1}(0.2 OD). Further results (including replicates) are provided in SI.

Finally, the implementation of the **G4-biotin** dendrimer as a control line for the detection of double-tagged amplicon of *E. coli* was also demonstrated. The double-tagged DNA from E. coli was obtained as previously described by our research group.[51,52] The procedure for the detection of *E. coli* by NALF is schematically described in Figure 5.

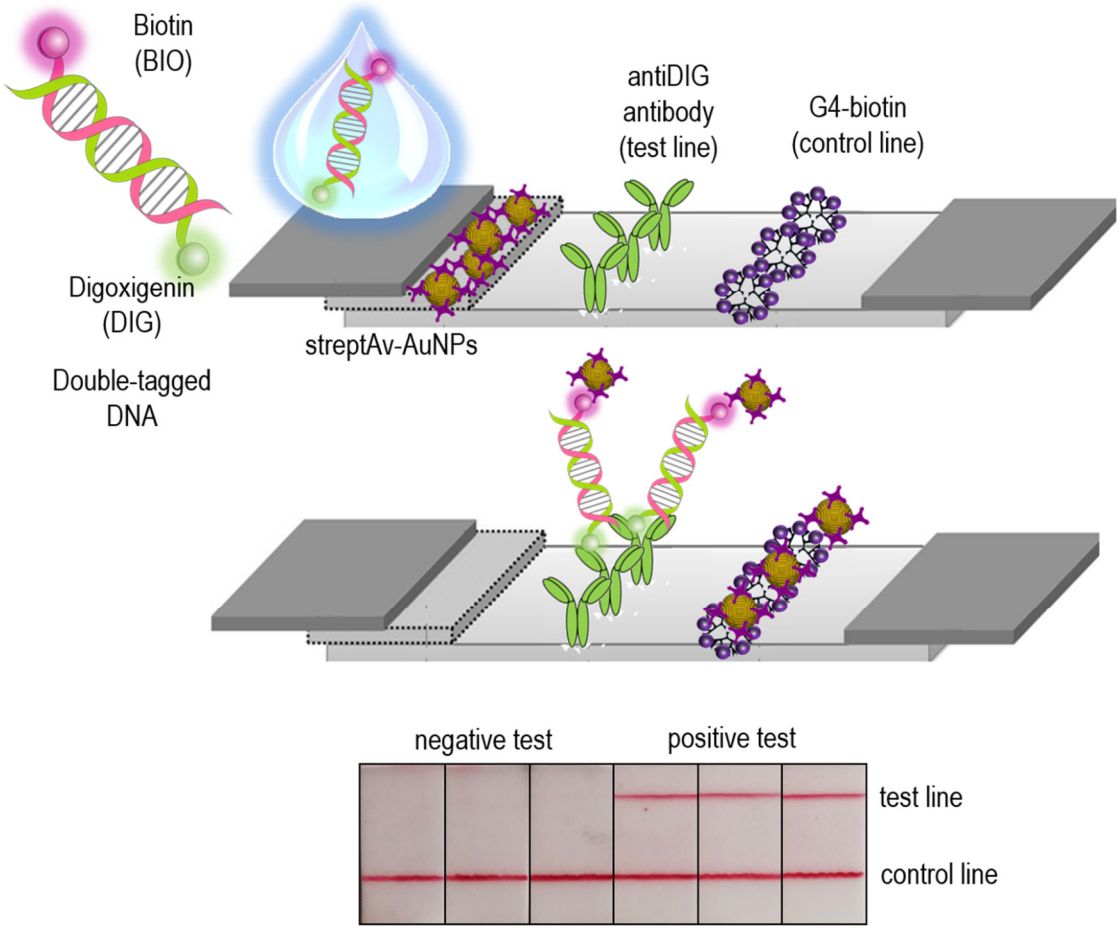


Figure 5. Up: General scheme of design of strips for a NALF test for the detection of double-tagged DNA, showing the details of the interaction between the biotinylated biomolecule (in this instance, the double-tagged DNA and the dendrimer) with streptAv-AuNPs used as signal generating system. Down: Images of strips of NALF tests for the detection of 300 ng mL^{-1} double-tagged amplicon from *E. coli* with streptAv-AuNPs at 5.90 10^{-11} mol L^{-1}(0.5 OD). Concentrations of dispensed solutions: 0.50 mg mL^{-1} for **G4-biotin** (control line), and 0.50 mg mL^{-1} for antiDIG antibody (test line). The negative control is also shown, n=3.

Upon the addition of the double-tagged DNA from *E.coli* (labelled each 5' end with biotin and digoxigenin), the streptAv-AuNPs thus reacted with the BIO-tag of the DNA from *E. Coli* (as shown in Figure 5). As the products moved along the strip, the streptAv-AuNPs/amplicons were captured by the specific antibodies adsorbed on the test line (antiDIG). A valid test was considered when the remaining streptAv-AuNPs reacted with a **G4-biotin** dendrimer as a positive control at the control line. The visual readout was thus achieved as well as the interpretation of the results. The specificity of the NALF was performed by challenging a positive sample as well as a negative control (n=3), as also shown in Figure 5. In this instance, only one line was observed as expected (the control line), since no DNA interacted in the test line.

CONCLUSIONS

Two phosphorus dendrimers of the first and four generation containing in their surface 12 and 96 biotin moieties (**G1-** and **G4-biotin**) have been synthesized and have shown an excellent performance when used as control line signal amplifiers in lateral flow assays, when using streptAv-AuNPs as a signal generating system. Smallest molecules such as free biotin and a model molecule containing similar structure of dendrimer branches and two biotin moieties, **3**, diffused in the same conditions. The hyperbranched structures of the dendrimers, the availability of numerous external biotin groups and their low solubility in buffer aqueous solution could be the responsible of their outstanding performance when compared with other biotinylated molecules such as **BSA-biotin** conjugate and **IgG-biotin**. In these cases, diffusion of the control line was observed. The behaviour of the dendrimers dispensed at different concentrations showed that **G1-biotin** provided more sensitive readout with the naked eye at lower concentrations (0.05-0.25 mg mL^{-1}), however both dendrimers could be used interchangeably working at concentration over 0.5 mg mL^{-1}. We recommend future users to deposit concentrations ranging 0.5 to 1 mg mL^{-1} of

dendrimers, either **G1-** or **G4-biotin**, in the control line in order to achieve clear valid results with the naked eye, as it is mandatory in this kind of tests. Nucleic acid lateral flow test prepared to detect *E. Coli* as a model has been performed using successfully the new dendrimers as signal amplifiers in the control line, showing an outstanding performance either in positive as well as in negative test. In addition to their proper performance, the dendrimers, especially **G1-biotin**, are cheap, very robust, can be stored for at least one month at room temperature, and are prepared using an animal-friendly procedure. These are the main advantages for their use in LFA as control line compared to more expensive biological molecules such as proteins (f. ex. BSA or antibodies) that should be conserved refrigerated and have expiration dates. This strategy will open a wide range of applications for different LFA.

ASSOCIATED CONTENT

Supporting Information. The supporting information is available free of charge on the ACS Publications website. Additional data on synthesis of precursor of compound **1**. NMR, IR and HRMS, AFM and TEM images, Intensity colour measurements (tables and graphics), preparation of lateral flow strips.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

LFA, lateral flow assay; DMF, dimethyl formamide; streptAv-AuNPs, InnovaCoat® GOLD 40 nm Streptavidin gold nanoparticles, BSA bovine serum albumine, Ig antibody.

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