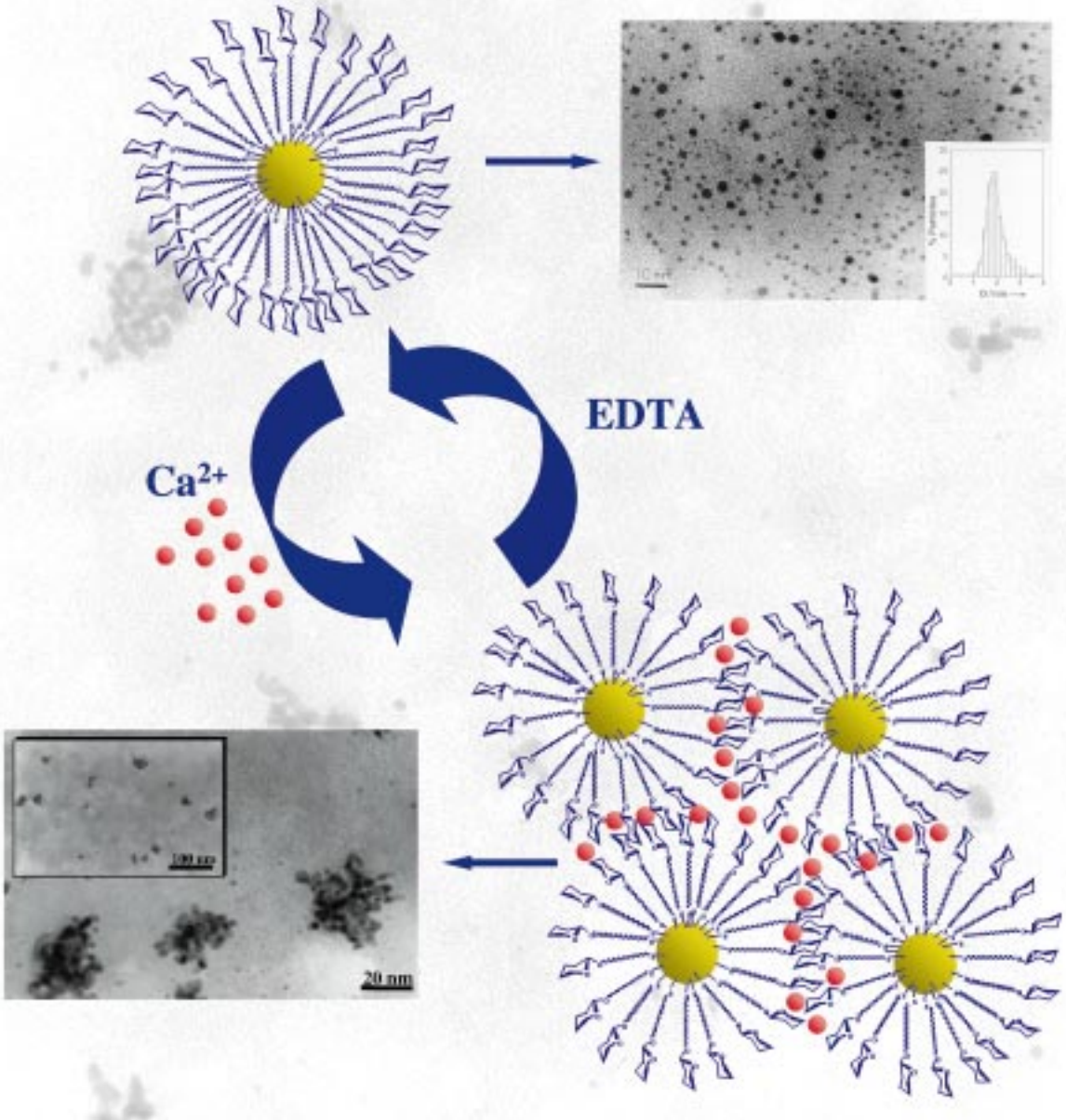
Glycosphingolipid clustering and interactions at the cell membrane can be modeled by gold glyconanoparticles prepared with biologically significant oligosaccharides.



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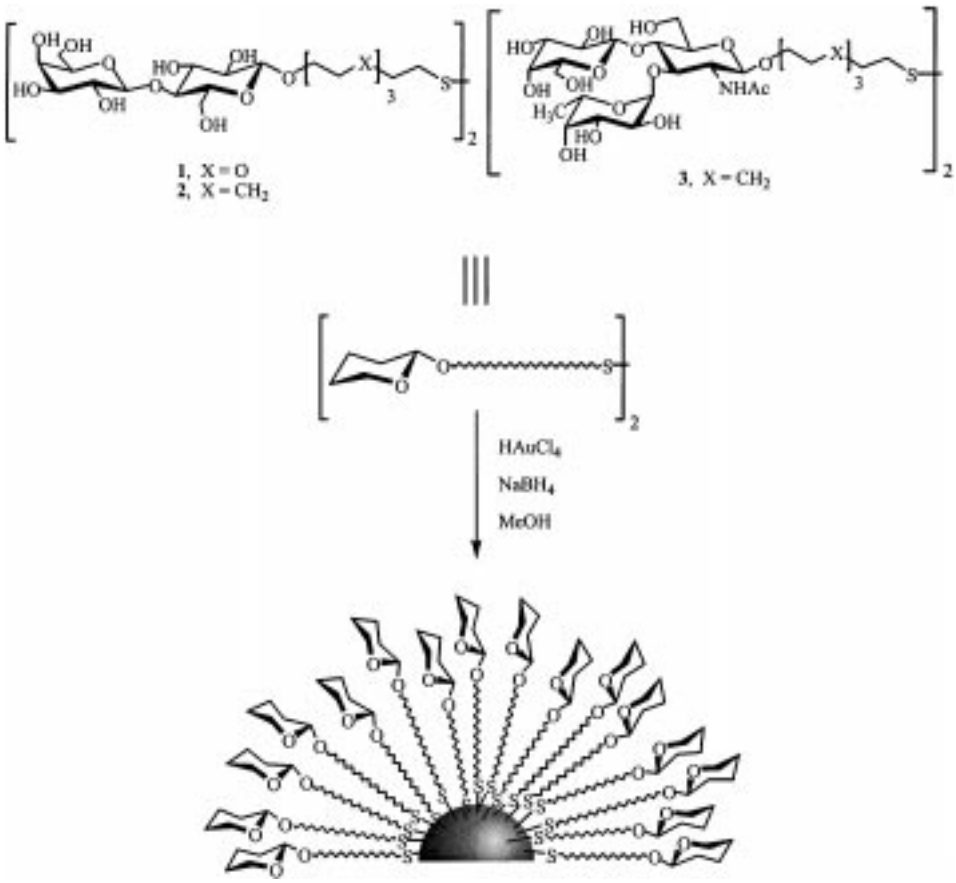
Gold Glyconanoparticles as Water-Soluble Polyvalent Models To Study Carbohydrate Interactions**

Jesús M. de la Fuente, Africa G. Barrientos, Teresa C. Rojas, Javier Rojo, Javier Cañada, Asunción Fernández, and Soledad Penadés*

Dedicated to Professor Manuel Martín-Lomas on the occasion of his 60th birthday

There is now evidence that in addition to protein–protein and protein–carbohydrate interactions, cells use attractive interactions between glycosphingolipid (GSL) microdomains as an initial step for adhesion and recognition.[1] GSL microdomains are functional units associated with cell adhesion, signal transduction, and other normal and pathological processes.[2] A homophilic carbohydrate–carbohydrate interaction between the Lewis^X antigen seems to be responsible for morula compaction,[3] and a heterotopic interaction between glycosphingolipid patches of GM3 and Gg_3 is involved in metastasis of melanoma cells in mice.[4] Characteristic features for this interaction are its strong dependency on divalent cations and its extremely low affinity, which is compensated in Nature by polyvalent presentation of the ligands.[5] These features make the study such interactions a real challenge. Herein we present a new water-soluble three-dimensional (3D) polyvalent model system for the study of carbohydrate interactions, this system consists of gold nanoclusters functionalized with neoglycoconjugates of biologically significant oligosaccharides. We illustrate the usefulness of these models with the preparation of gold nanoparticles functionalized with the disaccharide lactose or the trisaccharide Lewis^X determinant and also demonstrate the selective self-recognition of the Lewis^X functionalized glyconanoparticles by carbohydrate–carbohydrate interactions. These glyconanoparticles provide a glycocalyx-like surface with globular shapes and a chemically well-defined composition to intervene in the cell–cell adhesion processes.

Recently several groups have prepared multivalent carbohydrate model systems,[6–8] that have been used to study carbohydrate–protein interactions. Of special interest are the two-dimensional (2D) arrays of glycoconjugates on gold surfaces using self-assembled monolayers.[9, 10] Spherical arrays of carbohydrates, as represented by glycodendrimers,[11] and liposomes[12] have also been employed as useful tools to investigate carbohydrate recognition processes.



Scheme 1. Preparation of gold (gray hemisphere) glyconanoparticles.

We now report a simple and versatile approach by which carbohydrates are linked to gold nanoparticles as a method for tailoring highly polyvalent water-soluble carbohydrate surfaces with globular shapes (Scheme 1). This approach opens the way to tailoring glyconanoparticles with a variety of carbohydrate ligands and with differing ligand density, providing a controlled model for investigating the influence of carbohydrate clustering[13, 14] and orientation effects[15] on their interactions with specific receptors.

Thiol-derivatized neoglycoconjugates **1**–**3** of two biologically significant oligosaccharides, the disaccharide lactose (Galβ(1→4)Glcβ1-OR) and the trisaccharide Le^X (Galβ(1→4)[Fucα(1→3)]GlcNAcβ1-OR), have been pre-

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pared to functionalize gold nanoparticles in situ. The synthesis of the disulfides **1**–**3** was carried out by glycosidation of the conveniently protected lactose and Le^X derivatives with 11-thioacetate-3,6,9-trioxa-undecanol (for **1**) and 11-thioacetate undecanol (for **2** and **3**) by using the trichloroacetimidate method.[16] Both linkers have been used to test the versatility of the method in the preparation of glyconanoparticles with different spacers. Compounds **1**–**3** were isolated as disulfide forms, and used in this form for the formation of the water-soluble gold-protected glyconanoparticles **1**-Au, **2**-Au, and **3**-Au which were obtained in methanol by using the procedure of Brust et al.[17] for the synthesis of monolayer-protected gold nanoclusters. A series of gold-protected nanoparticles, soluble in organic solvents, has recently been prepared,[18–20] however, few examples have been reported of water-soluble gold nanoclusters.[21–23] The *lacto*-Au and Le^x-Au glyconanoparticles are stable, water soluble, and can be manipulated as a water-soluble biological macromolecule. They were purified by dialysis or centrifugal filtering and characterized by ^{1}H NMR, UV/Vis, and FT-infrared (IR) spectroscopy, elemental analysis, and transmission electron microscopy (TEM).

Figure 1 shows TEM images and core size-distribution histograms for **2**-Au and **3**-Au. A mean diameter of 1.8 nm was found for the gold core of the functionalized nanoparticles. Elemental analyses for **1**-Au, **2**-Au, and **3**-Au confirm a ratio of 63 (*lacto*-**1**), 70 (*lacto*-**2**), and 97 (Le^X-**3**) molecules per 201 gold atoms, in accord with data from previous work[21] for alkanethiol-protected nanoclusters of 2 nm mean diameter. These analyses correspond to molecular weights of 73, 76, and 109 kD, respectively. The aqueous solutions of the nanoparticles remained transparent for months and no agglomeration was detected by the naked eye and TEM examination.

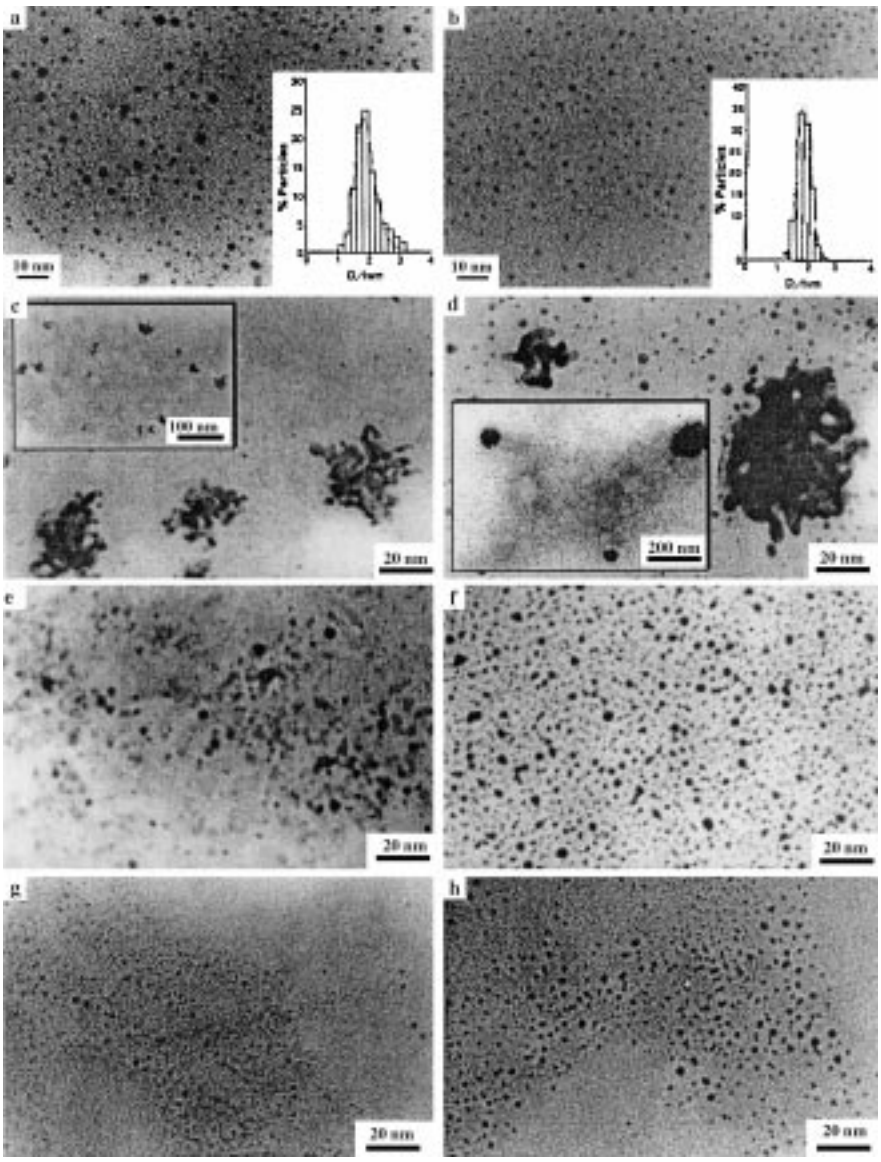

Figure 1. Transmission electron micrographs and core size-distribution histograms (insets in a, b) of: a) **3**-Au (0.1 mg mL^{-1} in water); b) **2**-Au (0.1 mg mL^{-1} in water); c) **3**-Au (0.1 mg mL^{-1} in 10 mM $CaCl_2$ solution); d) **3**-Au (0.9 mg mL^{-1} in 10 mM $CaCl_2$ solution); e) the same as (c) + EDTA; f) the same as (d) + EDTA; g) **2**-Au (0.1 mg mL^{-1} in 10 mM $CaCl_2$ solution); h) **2**-Au (0.9 mg mL^{-1} in 10 mM $CaCl_2$ solution); EDTA = ethylendiaminetetraacetate.

An important point for the application of our model to the investigation of the in vivo cell-adhesion processes, is the stability to enzymatic degradation. The steric crowding of the carbohydrate moiety on the nanoparticle surface may inhibit its recognition and degradation by enzymes.[24] We have tested the enzymatic hydrolysis of **1** and **2** and their corresponding nanoclusters, **1**-Au and **2**-Au, with β-galactosidase of *E. coli*. The enzyme processes **1** and **2** at a level comparable to that of lactose itself, while the hydrolysis of the **1**-Au and **2**-Au nanoparticles, under the same conditions, was barely detectable (< 3% relative to the enzymatic activity with the free ligands **1** and **2**).

The glyconanoparticles have been used as 3D models to mimic GSL clustering at the cell surface and to investigate the existence of carbohydrate–carbohydrate interactions in solution. Divalent cations seem to be essential in this interaction.[1] The aggregation of the *lacto*-**2**-Au and Le^X-**3**-Au nanoparticles has been studied in the presence and absence of calcium cations. TEM has also allowed us to observe the self-interaction events between the glyconanoparticles in water and calcium solutions. The *lacto*-Au and the Le^X-Au nanoparticles were dissolved at different concentrations (0.1 mg and 0.9 mg mL^{-1} for the *lacto*- and 0.1, 0.3, 0.5, and 0.9 mg mL^{-1} for the Le^X-Au nanoparticles) in 10 mM $CaCl_2$ solution, left overnight and aliquots of the solutions were then observed under the microscope. Comparison of the TEM micrographs obtained in water (Figure 1a, b) with those in calcium solution (Figure 1c, d, g, h) reveals clear differences between the *lacto* and the Le^X samples. The Le^X-Au nanoparticles in calcium solution show 3D aggregates at all

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concentrations (Figure 1c, d), while the *lacto*-Au nanoparticles remain dispersed as in the water solution (Figure 1g, h). The interaction of two Le^X-Au nanoparticles will result in the presentation of more carbohydrate epitopes at the surface allowing further aggregation in the presence of calcium. At a concentration of 1 mg mL^{-1} of **3**-Au the mean diameter of the aggregates reaches 100 nm.

Le^X–antigen aggregates arise from self-recognition between Le^X molecules in the presence of Ca^{2+} ions and not the result of nonspecific interactions with the salt. This notion is supported in that: first, salts usually induce flocculation processes in gold nanoclusters. However, neither the Le^X-Au nor the *lacto*-Au nanoparticles flocculate in water or calcium solution even after several months. Second, the *lacto*-Au nanoparticles under the same conditions did not show any aggregation. Third, removal of the calcium cations from the Le^X solution by adding EDTA results in the dispersion of the Le^X aggregates and the isolated nanodots were once again observed by TEM (Figure 1e, f).

These results indicate that only specific binding events between Ca^{2+} ions and the Le^X molecules dictate the self-aggregation process and confirm the Le^X determinant as a homophilic adhesion molecule. Measurements by atomic force microscopy (AFM) of the adhesion forces between self-assembled monolayers of the Le^X derivative **3** have also confirmed the Le^X antigen as an adhesion molecule.[28]

In conclusion, the glyconanoparticle approach presented here provides a versatile strategy to prepare a great variety of water-soluble highly polyvalent carbohydrate arrays with globular shapes by using a simple synthetic method. The simplicity of the preparation makes the glyconanoparticles a competitive tool in the study of polyvalency in carbohydrate interactions in solution. This strategy also allows us to prepare hybrid glyconanoparticles made up of carbohydrates and other molecules (biotin, fluorescent probes, peptides, etc.).

We have demonstrated the selective ability of the Le^X determinant for self-recognition in aqueous solution,[30] supporting the proposal that specific interactions between carbohydrates are possible and may represent an additional mechanism for cell adhesion and recognition. Previously, by the use of synthetic receptors, we have also demonstrated that stabilizing interactions between carbohydrate surfaces exist in water.[25, 26] The glyconanoparticles may also be considered to be a convenient tool to intervene in cell–cell adhesion and recognition processes where carbohydrates are involved. We are presently preparing other glyconanoparticles with other carbohydrate antigens. The application of the *lacto*-Au and Le^X-Au nanoparticles to interfere anti-adhesion therapy is presently underway. Preliminary results indicate that the *lacto*-nanoparticles inhibit in vivo lung metastasis of melanoma cells in mice.[29]

Experimental Section

Preparation of glyconanoparticles: A solution of disulfide **1**, **2**, or **3** (0.012 M, 5.5 equiv) in MeOH (5 mL) was added to a solution of tetrachloroauric acid (0.025 M, 1 equiv) in water (0.4 mL). An aqueous solution of $NaBH_4$ (1M, 22 equiv) was added in small portions with rapid stirring. The black suspension formed was stirred for an additional 2 h and the solvent then removed under vacuum. The glyconanoparticles are insoluble in MeOH but soluble in water (more than 1 mg/100 μL). Purification was performed by centrifugal filtration: crude product (50 mg) was dissolved in water (5 mL) NANOpure. This solution was loaded into a centrifugal filter device (CENTRIPLUS YM30, MICROCON, MWCO = 30000), and was subjected to centrifugation (3000 × g, 40 min). The dark glyconanoparticle solution was washed with MeOH and the process repeated several times until the starting material could no longer be detected by thin layer chromatography (TLC). The glyconanoparticles were dissolved in water and lyophilized. The products obtained were free of salts and starting material (absence of signals from disulfide and Na^+ ions in ^1H and ^{23}Na NMR spectroscopy).

Transmission electron microscopy: Examination of the samples was carried out with a Philips CM200 microscope working at 200 kV. A single drop of the aqueous solutions of the gold glyconanoparticles was placed onto a copper grid coated with a carbon film. The grid was left to dry in air for several hours at room temperature. Particle size distribution of the Au clusters was evaluated from several micrographs using an automatic image analyser. The number of particles selected for consideration was around 400, which resulted in stable size-distribution statistic. The same procedure was used for the calcium solutions. To remove calcium cations, EDTA solution was added to samples (final concentration in EDTA 50 mM). Solutions were loaded into a centrifugal filter device and were subject to centrifugation. The residues were dissolved in water (the original concentrations were kept) and a drop was place onto a copper grid.

Enzyme assays: Solutions of **1**, **2**, or the corresponding glyconanoparticles **1**-Au and **2**-Au (2 mM lactose equivalents) in a buffer solution (pH 7.2, sodium phosphate 50 mM, $MgCl_2$ 1 mM) were treated with *E. coli* β-galactosidase (SIGMA, 0.01 to 0.1 mg protein per mL). The galactose produced was measured by means of a standard enzymatic coupled assay with galactose deshydrogenase.[27]

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Electronic Transduction of Polymerase or Reverse Transcriptase Induced Replication Processes on Surfaces: Highly Sensitive and Specific Detection of Viral Genomes**

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Dedicated to Professor André M. Braun on the occasion of his 60th birthday

The detection of pathogens or autosomal recessive diseases is one of the future challenges of medicine and diagnostics.[1] Sensitive gene detection is accomplished by the polymerase chain reaction (PCR) amplification or secondary signal amplification routes.[2, 3] Major goals in future gene analysis include the parallel detection of a variety of pathogens and their quantitative assay. Inherent limitations of PCR prohibit the application of the method for quantitative and parallel high-throughput analyses. Microarrays of DNA have attracted substantial research efforts for the simultaneous analysis of genetic materials. In most of these systems the analyte samples are amplified by PCR cycles, and the microarrays act as a sensing interface that lacks amplification capabilities.[4, 5] Herein we address the development of ultrasensitive DNA-detection methods where in situ amplification proceeds on functionalized surfaces (electrodes or piezoelectric crystals) and the detection process is electronically transduced. The method enables the quantitative analysis of viral DNA and may be adopted for parallel analyses on arrays. Previous reports addressed the electrochemical[6–9] or microgravimetric,[10] quartz-crystal microbalance detection (QCM) of DNA. Several recent studies reported attempts to amplify DNA sensing processes: Dendritic, hyperbranched, oligonucleotides were employed to enhance the binding of DNA to electrodes.[11] The biocatalyzed precipitation of an insoluble product on the electronic transducer, that follows the primary hybridization between the analyte DNA and the probe oligonucleotide, was used to amplify the sensing event.[12] Also, labeled liposomes were employed as micromembrane interfaces that amplify the primary DNA-sensing events by their association to the probe-oligonucleotide/DNA-analyte complex generated on the transducer.[13, 14] Similarly, dendritic-type amplification of the analysis of a target DNA was accomplished by the use of oligonucleotide-functionalized Au-nanoparticles.[15] Faradaic impedance spectroscopy or frequency changes of the piezoelectric crystal, were used to transduce the different amplified sensing processes. Here we report a novel ultrasensitive method for the electronic transduction of the detection of viral nucleic acids. We demonstrate the surface polymerase-induced or reverse transcriptase stimulated formation of double-stranded DNA or RNA on the transducer, and the secondary amplification of the sensing process by the biocatalyzed precipitation of an insoluble product. Electrochemical and microgravimetric QCM methods are used as electronic transduction means for the DNA detection. The process is exemplified by the analysis of the M13 mp8 (M13ϕ) DNA (ca. 300 copies per 10 μL) and of the RNA of vesicular stomatitis virus (VSV; ca. 60 copies per 10 μL).

The method for analysis of the target is depicted in Scheme 1. The primer thiolated oligonucleotide **1**, complementary to a segment of the target M13 mp8 DNA, is assembled on an Au-electrode or an Au-quartz crystal through a thiol functional group.[16, 17] The sensing interface is then treated with the analyte DNA of M13 mp8 (+) strand, and the resulting complex on the transducer is treated with dATP, dGTP, dTTP, dCTP, and biotinylated-dCTP (ratio 1:1:1:2/3:1/3, nucleotides concentration of 1 mM) in the presence of DNA polymerase I, Klenow fragment (20 U mL^{-1}).[18] Polymerization and the formation of a double-stranded assembly with the target DNA is anticipated to provide the first amplification step of the analysis of the viral DNA. Polymerase introduces biotin tags to the double-stranded assembly, thus providing a high number of docking sites for the binding of the avidin – alkaline-phosphatase conjugate. The associated enzyme biocatalyzes the oxidative hydrolysis of 5-bromo-4-chloro-3-indolyl phosphate (**2**) to form the insoluble indigo product **3**, that precipitates on the transducer, thus providing a second amplification step for the analysis of the target DNA.[19] The synthesized strand on the electrode is anticipated to attract a positively charged redox label that can be assayed by chronocoulometry.[20] This approach enables us to monitor the polymerization process continuously. The negatively charged double-stranded assem-

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