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Ultrasensitive nanostructured immunosensor for stem and carcinoma cell pluripotency gatekeeper protein NANOG

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Abstract

Aims—To develop an immunosensor for ultrasensitive detection of the NANOG protein. NANOG regulates pluripotency in stem cells and some cancer cells. This article reports the first electrochemical immunosensor for ultrasensitive detection and absolute quantification of the NANOG protein. The sensor features dense capture antibody-coated gold nanoparticle layers on a pyrolytic graphite underlayer.

Materials & methods—Two separate multilabel detection strategies were used to achieve moderate and ultra-high sensitivity.

Results—Good sensitivity was achieved for NANOG over the concentration range 0.1–160 pg/ml. The moderate sensitivity approach gave a detection limit of 25 pg/ml, while the ultrasensitive method achieved a 250-fold lower detection limit of 0.1 pg/ml. Amounts of NANOG detected in human embryonic stem cell lysates correlated well with qualitative western blots and mRNA expression.

Conclusion—The electrochemical gold nanoparticle immunosensor is suitable for measuring NANOG protein expression in stem and carcinoma cell tissue lysates at very low levels.

Keywords

cancer biomarker; electrochemical immunosensor; gold nanoparticle; magnetic particle; NANOG; pluripotency; stem cell

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Accurate, sensitive detection of proteins is a central need for progress in biomedical science. Proteins that are upregulated or downregulated correlated with changes in cell differentiation or at the onset of disease hold significant value in diagnostics and research [1–3]. Development of devices and protocols to measure these proteins sensitively, cheaply and accurately is currently the subject of intense research activity, especially with regard to cancer biomarkers [2,4–7].

NANOG is a key transcription factor first found to be expressed specifically in mouse embryonic stem cells (ESCs). The protein NANOG in mouse and human stem cells is a critical gatekeeper for maintaining pluripotency [8–11], that is, the ability of a single cell to differentiate into any of the three germ layers (endoderm, mesoderm or ectoderm). When NANOG is downregulated in human embryonic stem cells, they differentiate [10]. Also, NANOG is overexpressed in germ cell, embryonal, breast and testicular carcinomas, and is thus a potentially valuable diagnostic biomarker for undifferentiated germ cell tumors [10,12–14]. A low level of NANOG transcriptional expression has also been detected in adult murine tissues [15]. Based on human-expressed sequence tag profiles, NANOG mRNA transcripts are expressed in embryonic tissue and two adult tissues, testis and blood. The NANOG expression in testis [16,17] may be due to the constant high turnover and formation of sperm, which are derived from a pool of adult stem cells present in this tissue. Kim *et al.* reported the expression of NANOG in bone marrow and cord blood cells [18], which may be correlated with the existence of circulating adult stem cells [19,20].

Given the critical general role of NANOG in regulating cell pluripotency and germ cell cancer development, accurate and precise methods for its detection should be a high priority. NANOG transcription can be analyzed by quantitative PCR techniques at the RNA level [21]. For correlating transcript expression to detecting NANOG protein, semiquantitative western blotting [22] and immunohistochemical approaches [23] are often employed. However, none of these techniques provide absolute levels of NANOG present in cells. Relative quantitation of NANOG can be obtained by a Taqman protein assay utilizing PCR amplification of an oligonucleotide label [24]. NANOG quantification can also be done by recently introduced human NANOG ELISA kits by Antigenix America (NY, USA) and CUSABIO Biotech Co. (DE, USA) with detection limits (DLs) of approximately 2 pg/ml. In this article, we report the first electrochemical immunosensor for the detection of NANOG protein and an assay protocol to quantify absolute levels of NANOG in cell lysates down to 0.1 pg/ml.

The new immunosensor described herein builds on our recently developed nanostructured electrochemical sensors for cancer biomarker proteins featuring single-wall nanotube forests or gold nanoparticle (AuNP) platforms coupled with multilabel enzyme-antibody particles. Chemically functionalized nanostructured surfaces provide high densities of accessible, attached capture antibodies to help enhance sensitivity [25]. These approaches have achieved sub-pg/ml detection of cancer biomarker proteins such as prostate-specific antigen (PSA) [26,27] and IL-6 [28,29] in serum. These sensors also detected PSA accurately in cancer patient serum and tissue lysates [26,27]. We used an array of four nanotube forest sensors for simultaneous accurate measurement of prostate cancer biomarkers PSA, prostate-specific membrane antigen, platelet factor-4 and IL-6 in cancer patient serum [30].

Antibody-loaded magnetic nanoparticles have been used previously in immunoassay protocols for offline analyte capture [31–34], a strategy that can greatly decrease nonspecific binding of interfering biomolecules. Using this approach with clustered magnetic particle (MP) labeling, we measured PSA in serum at a DL of 10 fg/ml in a surface plasmon resonance immunoassay [33]. Using MPs massively labeled with enzymes, we detected IL-8 in diluted serum and cancer cell conditioned media with an ultralow DL of 1.0 fg/ml [35].

In this paper, we report the application of our immunosensor strategies to develop a new sensor for quantitative and sensitive measurements of NANOG in cell lysates. We combined enzyme-labeled secondary antibody (Ab₂) protocols with the AuNP sensor platform in sandwich immunoassays. Briefly, pyrolytic graphite sensor disk electrodes are coated with dense films of 5-nm AuNPs decorated with antibodies that capture NANOG protein very efficiently from a liquid sample. Two separate multilabel detection strategies were used to achieve sensitivity over a broad range of concentrations. In a moderate sensitivity approach, Ab₂-biotin-streptavidin-horseradish peroxidase (HRP) bioconjugates (Figure 1A) were used to bind to NANOG captured on the sensor surface to provide 14–16 labels per antigen [28]. In a second, ultrasensitive approach (Figure 1B), we used streptavidin-coated magnetic beads conjugated with biotinylated Ab₂ and biotinylated-HRP (400,000 HRPs per particle) in the detection step. The sensor detected NANOG with DL of 100 fg/ml (3 fM). Sensor validation was confirmed by successful measurements of NANOG in various cell lysates with good correlation to western blots and relative RNA expression levels.

Materials & methods

Chemicals & materials

Monoclonal (mouse) primary NANOG antibody (Ab₁ ab76586), biotinylated rabbit polyclonal NANOG antibody (Ab₂ ab84231), NANOG protein (ab50053) and streptavidin protein (HRP ab7403) were obtained from Abcam (Cambridge, UK). Tissue lysate samples were obtained from Protein Biotechnologies (CA, USA). L-gluthathione reduced (99% H₂AuCl₄ × 3H₂O [99.9%]), sodium borohydride (99%) poly(diallyldimethylammonium chloride) (MW 20%) and bovine serum albumin (BSA) were from Sigma-Aldrich (MO, USA). Streptavidin coupled MPs (Dynabeads® MyOne Streptavidin T1), and biotinylated HRP were from Invitrogen (CA, USA). 1-(3-(dimethylamino) propyl)-3-ethylcarbodiimide hydrochloride and N-hydroxysulfosuccinimide, from Sigma-Aldrich, were dissolved in water immediately before use. Hydrogen peroxide (H₂O₂; 30%) was from Fisher (NJ, USA). Tween-20 was from Sigma-Aldrich. Immunoreagents were dissolved in pH 7.0 phosphate-buffered saline (PBS 0.01 M in phosphate, 0.14 M NaCl, 2.7 mM KCl) unless otherwise noted.

Instrumentation

A CHI 660 electrochemical workstation (CH Instruments, TX, USA) was used for amperometry at ambient temperature (22 ± 2°C). Rotating disk amperometry was done at –0.3 V versus standard calomel reference electrode with electrodes rotating at 3000 rpm.

Preparation of MP-Ab₂-HRP conjugates

Supplier (Invitrogen) protocol was followed to conjugate the biotinylated-Ab₂ and biotinylated-HRP with MPs. A total of 20 µl of MPs (100 mg/ml) were washed twice with 0.1% BSA in PBS buffer (pH 7.4) and resuspended to the same volume. Then 50 µl of biotinylated antibodies (Ab₂ 5 µg/ml) and 30 µl of biotinylated HRP (2.5 mg/ml) were added to the MPs and incubated at room temperature for 30 min using a slow tilt rotator. MP-Ab₂-HRP conjugates were separated magnetically and washed with 0.1% BSA in PBS four-times and later reconstituted in PBS (pH 7.4), and stored at 4°C till further use.

The number of HRP labels per MP was calculated from the measured enzyme activity using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) assay [36] as reactant to be 4 (± 0.9) × 10⁵ per bead, estimated on the total number of magnetic beads in 1 ml, 9 × 10⁹.

Fabrication of the immunosensor

The AuNP sensor platform was fabricated using layer-by-layer alternate electrostatic adsorption [37,38]. Abraded disks ($A = 0.14 \text{ cm}^2$) of pyrolytic graphite (GE Ceramics, DE, USA) embedded in heat-shrinkable tubing and mounted on steel rods were used as the base electrodes onto which the immunosensors were fabricated. Initially, a 0.5-nm layer of cationic poly(dimethyldiallylammonium) chloride was adsorbed from a 20- μl drop placed onto the (0.14 cm^2) pyrolytic graphite disk electrode, followed by washing with water. Next, a negatively charged layer of glutathione-decorated 5 nm AuNPs was deposited from a dispersion onto the poly (diallyldimethylammonium chloride)-coated electrode [27]. The AuNP layer caused a 29% increase in surface area over the underlying pyrolytic graphite surface. Surface area was estimated from cyclic voltammetry using the Randles–Sevcik equation [39] and slope of peak currents of soluble 1 mM ferrocyanide in 0.1 M KCl versus square root of scan rate.

Antibodies for NANOG were anchored to the AuNP-glutathione layer by placing 30 μl of freshly prepared 400 mM ethylcarbodiimide hydrochloride and 100 mM *N*-hydroxysulfosuccinimide in water onto AuNP electrodes for 10 min, rinsing with water and then incubating the activated electrode surface for 2.5 h with 20 μl of a 3.3 pmol solution (25 $\mu\text{g}/\text{ml}$) of primary NANOG antibody in pH 7.0 PBS. Unbound antibody was removed by rinsing thoroughly with 0.05% Tween-20 in PBS and then PBS for 2 min each. To block the free antibody binding sites on the sensor surface, the immunosensors were incubated with 10 μl of 2% BSA and 0.05% Tween-20 in PBS for 45 min at 37°C, followed by washing with 0.05% Tween-20 in PBS and then PBS for 2 min each.

Sensor operation

For the actual NANOG measurements, the immunosensors were incubated with 10 μl of NANOG standard solutions in pH 7.0 PBS buffer or cell/tissue lysates for 1 h at 37°C. After the washing steps, the sensors were exposed to 10 μl of 1 $\mu\text{g}/\text{ml}$ biotinylated-Ab₂ conjugates in buffer containing 0.1% BSA in pH 7.4 PBS buffer for 1 h, followed by washing with 0.05% Tween-20 in PBS and PBS buffer for 2 min each.

Using the moderate sensitivity protocol (Figure 1A), the sensors were next incubated with 10 μl of streptavidin-HRP for 30 min, followed by thorough washing with 0.05% Tween-20 in PBS and then PBS for 2 min each. For the high-sensitivity protocol (Figure 1B), the sensors with captured NANOG from standards or cell/tissue lysates were incubated with multilabel MP conjugates (conjugated with biotinylated-Ab₂ and biotinylated-HRP) for 1 h. Finally, the completed immunosensors were placed in an electrochemical cell containing 1 mM hydroquinone in PBS buffer. Rotating-disk electrode amperometry was carried out by controlling the potential at -0.3 V versus saturated calomel electrode injecting H_2O_2 to obtain 0.1 mM concentration and measuring the amperometric reduction current. When using MP bioconjugates, where the concentration of hydroquinone was 0.25 mM, injected H_2O_2 was adjusted to reach a 0.05 mM concentration.

Sodium dodecyl sulfate gel electrophoresis and immunoblotting

Total cell lysates were obtained from human embryonic stem cells (hESCs) following manufacturer's instructions (Ambion, TX, USA). In total, 7 μg of proteins or various concentrations of pure NANOG protein were run on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred onto nitrocellulose membrane (Bio-Rad). Membranes were washed with 1X Tris-buffered saline containing 0.05% Tween-20, blocked in 5% milk in 1X Tris-buffered saline containing 0.05% Tween-20 and incubated with respective primary antibodies at conditions recommended by the manufacturers (Abcam NANOG monoclonal antibody, ab76586, 1:500 and β -actin, Cell Signaling 1:1000).

After washing with 1X Tris-buffered saline containing 0.05% Tween-20, membranes were incubated with respective HRP-conjugated Ab₂. Signals were detected with LumiGLO chemiluminescent reagent (Cell Signaling Technology, MA, USA).

Results

Sensor characteristics & detection limits

Nearly complete coverage of AuNPs on the sensor surface and attachment of a high density of capture antibodies have been confirmed previously for this sensor design by atomic force microscopy [27]. Using sensors with antibodies attached, a drop of NANOG standard solution or cell lysate is placed onto the sensor, which is then incubated and washed (see 'Material & methods' section). Next, a HRP-labeled Ab₂ or HRP-antibody-MP is used to bind to the captured NANOG to enable the detection step. At this point, the sensor is washed to minimize nonspecific binding and placed into the measuring cell containing buffer, then rotated while a potential of -0.3 V versus saturated calomel electrode is applied. Injection of H₂O₂ into the buffer solution activates the HRP labels to ferryloxy species that then get reduced by a hydroquinone mediator also present in the solution. The role of hydroquinone is to shuttle electrons from the underlying sensor surface to the HRP labels to complete the catalytic cycle and provide an amperometric steady-state current proportional to the amount of NANOG in the sample. Entire assays from placement of the sample on sensor to signal measurement takes approximately 135 min for high sensitivity and 155 min for moderate sensitivity.

Responses for the immunosensors to NANOG protein in the pg/ml range using the moderate sensitivity Ab₂-biotin-streptavidin-HRP labels (Figure 1A) show a fast rise to the steady-state currents in Figure 2A upon injection of H₂O₂. The control with no NANOG gives a finite steady-state current due to a small amount of residual nonspecific binding of the labeled Ab₂ plus residual direct reduction of the H₂O₂. Steady-state responses increased from 25 to 150 pg/ml NANOG (Figure 2B) with a sensitivity as the slope of the calibration graph of 4.6 nA·ml/cm²/pg NANOG. The DL as the blank response plus three-times the average noise was 25 pg/ml NANOG.

Standardization results for the high sensitivity AuNP immunosensors featuring massively labeled streptavidin-MP-biotin Ab₂-biotin-HRP bioconjugates (Figure 1B) are shown in Figure 3. Good quality, low signal/noise traces with well defined steady-state signals were obtained. Calibration graphs are shown in a linear-log format that covers the range 0.1–20 pg ml⁻¹ as well as a plot of the lowest concentration range. Despite the nonlinearity of these calibrations, sensitivity of the sensor is still applicable for quantitation of NANOG in this range. Using the streptavidin-MP-biotin Ab₂-biotin-HRP bioconjugates, the DL of 100 fg/ml was 250-fold better than that measured with the Ab₂-biotin-streptavidin-HRP system, mainly due to significant signal amplification brought about by the 400,000 HRP labels on each magnetic bead. Sensitivity of the immunosensor in the lower concentration range was 171 nA·ml/cm²/pg NANOG, 175-fold better than the moderate sensitivity approach. Sensitivity was estimated by dividing the slope of the current versus NANOG (from a linear fit in low concentration range [Chikkaveeraiah B, Soldà A, Rusling J, Unpublished Data]) by area of the working electrode (0.14 cm²). Standard deviations show that reproducibility was a little worse than that in the higher concentration range but still acceptable for such low levels. Even a small amount of nonspecific binding of the MP bioconjugates in the amplification strategy causes a significant blank signal as the signal is due to approximately 400,000 HRP labels on the MP, as seen for the zero NANOG blank (Figure 3A).

Quantitative NANOG detection in cell lysates

We evaluated the sensor for determining levels of NANOG protein in cell lysates of testis tissue [17], with liver tissue as a negative control and hESCs as positive controls. We found that NANOG protein expression normalized to total cellular protein was six-times higher in hESCs compared with the testis tissue (Figure 4) and the negative control was very low in NANOG.

Because NANOG expression is known to be downregulated under differentiating conditions [8,10,16], we also examined its expression in differentiating hESCs at different times. The spontaneous method of differentiation was used, which involves culturing the hESCs in the stem cell medium devoid of FGF-2 [40,41]. Our data, as expected, showed that NANOG expression decreased significantly with increasing differentiation time. We confirmed this by sodium dodecyl sulfate-gel electrophoresis and immunoblotting with NANOG-specific antibody that showed a similar trend in decreased intensity with increasing differentiation of the band corresponding to NANOG (Figure 5). However, the differences found by the immunosensor are much more apparent than the western blot analyses, with its low sensitivity being apparent in the relative similarity of the undifferentiated and differentiated samples in Figure 5, compared with more precisely determined differences seen in the immunosensor responses in Figure 4.

The regulation of NANOG expression can be detected at the transcriptional level by quantifying the NANOG transcripts with real-time PCR. Hence, we correlated our NANOG protein expression data with the relative mRNA expression level in the differentiating hESCs. The NANOG transcript expression showed a 21% decrease at D5 compared with control hESCs followed by a 52% decrease at D10. This shows qualitative correlation with immunosensor results (Figure 4) of 40% decrease in actual amounts of NANOG at D5 and 70% decrease in NANOG at D10. Thus, the cellular machinery responsible for NANOG transcripts expression followed a similar trend of decline with the directly observed NANOG levels measured by the immunosensor.

Discussion

The results document the fabrication, characterization and practical evaluation of the first nanostructured immunosensor for direct quantitative measurement of NANOG. This achievement is highly significant due to the key role of this protein in regulating stem and carcinoma cell differentiation. Using the new immunosensor and simple, inexpensive protocols, absolute amounts of NANOG were determined in cell lysates for the first time. The two protocols employed, covering moderate and high sensitivity, provide viable NANOG detection from approximately 0.1 to 150 pg/ml, with higher concentrations becoming accessible by simple sample dilution. The highest sensitivity is enabled by combining the nanostructured sensor platform with high antibody loadings on MPs with 400,000 HRP labels, which leads to an excellent limit of detection of 100 fg/ml.

Proof-of-concept data were presented for applicability of the new immunosensor to quantitative detection of NANOG in cell lysates. Results for lysates of hESC, testes tissue and control liver tissue gave results consistent with expected levels of NANOG, as well as with western blot results. Similarly, decreased NANOG measured in differentiating hESC tissue was consistent with independently measured relative mRNA expression levels.

Conclusion & future perspective

We have developed and validated the first electrochemical immunosensor for the quantitative ultrasensitive detection of NANOG at sub-pg/ml (fM) levels. Good accuracy of

the immunosensor was supported by good correlations between NANOG levels obtained in tissue lysates of hESC and testes cells with those estimated by semiquantitative western blot analysis and with mRNA expression levels. Good selectivity was confirmed by results from the same lysates, which contain many potentially interfering proteins. The main advantage of these immunosensors is ease of fabrication, low cost, ultrasensitivity, speed and viable quantification of NANOG.

At present there is no other method to measure absolute amounts of NANOG in cell lysates at such low levels without using PCR amplification, so the new immunosensor fulfills an important niche for direct quantitative NANOG detection. This sensor is expected to be a valuable tool in stem cell research and future cancer diagnostics. In addition, the technology is amenable to incorporation in microfluidic systems [34] that could detect NANOG and other biomarker proteins simultaneously. Such a device could provide semi-automated, multiplexed analyses of stem and carcinoma cell lysates, possibly with higher sensitivity and shorter analysis times, and expand the value of the applications described above.

Executive summary

- A new nanostructured electrochemical immunosensor was developed for the pluripotency gatekeeper protein NANOG that provides absolute quantitative detection in cell lysates.
- NANOG detection can be achieved in the 0.1–150 pg/ml range, with higher levels accessible by sample dilution.
- Ultrasensitive sensor response is achieved by combining a nanostructured surface with a massively labeled detection particle.
- The NANOG immunosensor provides a valuable quantitative tool for stem cell research and future cancer diagnostics.

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Figure 1. Alternative strategies for electrochemical immunosensors featuring a gold nanoparticles sensor platform with attached antibodies that capture the protein analyte
(A) Immunosensor after incubating with biotinylated secondary antibody and streptavidin-horseradish peroxidase (HRP) complex, providing 14–16 HRP labels. **(B)** Immunosensor after incubating with streptavidin-coated magnetic particles conjugated with biotinylated secondary antibody and biotinylated HRP (400,000 labels/particle). Amperometric signals are developed by injecting hydrogen peroxide at -0.3 V with hydroquinone mediator into the buffer.

AuNP: Gold nanoparticles; PDDA: Poly(diallyldimethylammonium chloride).

BSA: Bovine serum albumin; PG: Pyrolytic graphite.

Figure 2. Immunosenor data using the moderate sensitivity protocol

(A) Amperometric responses of gold nanoparticle immunosensor with attached capture antibody incubated with NANOG protein standards (pg/ml) in 10 µl buffer for 1 h after treating with 20 µl of 2% bovine serum albumin for 45 min, followed by the addition of 10 µl biotinylated enzyme-labeled secondary antibody in 0.1% bovine serum albumin and 0.05% Tween-20 for 45 min, and later incubating with streptavidin–horseradish peroxidase for 30 min. The current profiles were recorded upon injection of H₂O₂ (0.1 mM), into a buffer solution containing the sensor with captured NANOG and detection antibodies, and 1 mM hydroquinone, with electrode potential at –0.3 V and a rotation speed of 3000 rpm. (B)

Immunoarray calibration plot showing the influence of NANOG concentration on the steady-state sensor current.

Figure 3. Immunoarray data using the high-sensitivity protocol

(A) Amperometric responses of gold nanoparticle immunosensor with attached capture antibody incubated with NANOG protein standards (pg/ml) in 10 μ l buffer for 1 h after treating with 20 μ l of 0.1% bovine serum albumin (20 mM phosphate buffered saline; pH 7.4) for 45 min, followed by addition of 10 μ l magnetic particles bioconjugates and incubation for 1 h. The current profiles were recorded upon injection of H_2O_2 (0.05 mM), into a buffer solution containing 0.25 mM hydroquinone, while keeping the electrode potential at -0.3 V and rotation speed at 3000 rpm. (B) Linear-log immunoarray calibration

plot showing the influence of the NANOG concentration on the steady-state current signals.
(C) Influence of very low NANOG concentrations on immunosensor steady-state signals.

Figure 4. Immunosensor assay results normalized for total protein for human embryonic stem cells, testis and liver tissue lysate samples

Labels at the bottom of the graph denote differentiated hESCs samples tested at different times.

D5: Day 5; D10: Day 10; D15: Day15; hESC: Human embryonic stem cells; L: Liver tissue; T: Testis tissue.

Figure 5. Western blot analyses of cell lysates of human embryonic stem cells at differentiation time points in days

(A) NANOG protein expression and (B) β -actin protein expression. Total protein (7 μ g) was run on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred onto nitrocellulose membranes and were initially probed with NANOG monoclonal antibody (ab76586; 1:500). This was followed by labeling of the nitrocellulose membrane with β -actin antibody to show equal loading of the protein samples. (C) Western blot analysis of pure NANOG protein (ab50053) probed with same NANOG antibody (ab76586).

hESC: Human embryonic stem cells.