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Sirtuin 1 evaluation with a novel immunoassay approach based on TiO_2–Au label and hyperbranched polymer hybrid

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ABSTRACT

Accurate and highly sensitive evaluation of the sirtuin 1 (SirT1) level is becoming increasingly important for understanding the contribution of SirT1 in metabolism pathways. Here, a novel electrochemical immunoassay of SirT1 based on crosslinked hyperbranched azo-polymer decorated with gold colloids (Au–HAP) as sensing platform and titanium dioxide (TiO_2)–Au nanocomposites to immobilize secondary antibody–horseradish peroxidase (Ab_2–HRP) as electrochemical labels has been designed. Greatly enhanced sensitivity was achieved by exploiting the excellent conductivity of Au nanoparticle, the amplification effect of Au–HAP and TiO_2–Au, and the favorable catalytic ability of HRP. The nanocomposites of Au–HAP and TiO_2–Au could attach numerous capture antibodies on the surface for significant immune recognition efficiency. Meanwhile, the TiO_2–Au-labeled Ab_2–HRP using an HRP–thionine–H_2O_2 (hydrogen peroxide) detection system could further induce signal readout. Under optimal conditions, the signal intensity was linearly related to the concentration of SirT1 in the range of 1–500 ng ml^{-1}, and the limit of detection was 0.28 ng ml^{-1}. The developed biosensor exhibits attractive performance for the analysis of SirT1, with rapid response, high sensitivity, and high accuracy, and could become a promising technique for protein detection.

Human sirtuin 1 (SirT1) belongs to the mammalian SirT family and is closely related to yeast Sir2. Interest in SirT1 has grown since the initial reports that the *Saccharomyces cerevisiae* and *Caenorhabditis elegans* orthologues mediate the effects of calorie restriction (CR) to extend the lifespan of these organisms [1]. Much research has indicated that SirT1 plays a role in a wide range of cellular processes, including metabolism, cell cycle, cell growth and differentiation, apoptosis, and cellular response to stress [2], and has been shown to reduce age-associated physiological changes in mice [3,4]. Taking stock of key numbers of SirT1 in cell and molecular biology enables back-of-the-envelope calculations that test and sharpen our understanding of cellular processes [5]. Therefore, to clarify the functions of SirT1, the development of effective methods for quantitative analysis of this protein has tremendous importance in clinical diagnosis and drug screening. Up to now, instrumental methods such as Western blot [6], enzyme-linked immunosorbent assay (ELISA) [7], and reverse transcription polymerase chain reaction (RT–PCR) [8] all have been used for the study of protein evaluation. However, most methods often necessitate sophisticated instrumentation and clinically unrealistic expense and time.

Immunoassay of proteins has become an important solution for the bioanalytical problems in various fields such as disease diagnosis. The increasing demand for the screening of diseases at their early stage requires the ultrasensitive detection of biologically relevant species at an extremely low level of expression [9,10]. Thus, great efforts currently have been centered on the development of novel and effective strategies to produce and amplify signal readout. Some successful signal amplification strategies include the employment of new redox-active probes, the integration of

Abbreviations used: SirT1, sirtuin 1; PNC, polymeric nanocomposite; TiO_2, titanium dioxide; Au, gold; HAP, hyperbranched azo-polymer; $Ab_1^{\#1}$, goat polyclonal anti-SirT1; $Ab_1^{\#2}$, rabbit polyclonal anti-SirT1; Ab_2, secondary antibody; HRP, horseradish peroxidase; NHS, *N*-hydroxysuccinimide; EDC, 1-ethyl-3-3(3-dimethylaminopropyl) carbodiimide; SPDP, *N*-succinimidyl-3-(2-pyridyldithiol) propionate; PTMP, pentaerythritol tetrakis(3-mercaptopropionate); THPC, tetrakis (hydroxymethyl) phosphonium chloride; BSA, bovine serum albumin; $HAuCl_4$, chloroauric acid; PBS, phosphate buffer solution; EDX, energy dispersive X-ray; SEM, scanning electron microscopy; TEM, transmission electron microscopy; ICP–AES, inductive coupled plasma–atomic emission spectroscopy; UV, ultraviolet; SCE, saturated calomel electrode; ITO, indium tin oxide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; PDOP, polydopamine; MACA, mercaptoacetic acid; H_2O_2, hydrogen peroxide; AFM, atomic force microscopy; EIS, electrochemical impedance spectroscopy; DPV, differential pulse voltammetry.

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enzyme-assisted labels, and the incorporation of nanomaterials to increase the load of electrochemical tags [11,12]. In spite of many important achievements, enormous efforts on efficient signal amplification approaches are still needed to focus on improving the analytical performance and broadening the applications for immunoassay. The polymeric nanocomposites (PNCs) of polymer, nanoparticles, and more components have drawn much attention for their obvious technical and scientific interests in many fields such as biosensing. The main advantages of PNCs as biomaterials are as follows. First, the polymer matrix with high functionality and processability can efficiently entrap and adsorb the species of interest such as biomolecules [13] and nanoparticles [14]. Second, nanoparticles with significant adsorbability and biocompatibility can provide PNCs with abundant sites to bind biomolecules and retain the bioactivity [15,16]. Third, the incorporation of plenty of nanoparticles can endow PNCs with diversified functions for developing biodevices involving catalysis, electronics, optics, and magnetics. Fourth, PNCs are cost-effective, stable, and convenient to prepare [17,18].

In this article, we report an investigation of a novel immunoassay approach using hyperbranched polymer hybrids cooperating with titanium dioxide–gold (TiO_2–Au) nanocomposite as electrochemical labels to assay SirT1. Hyperbranched polymers become very intriguing with regard to design, synthesis, and functional property because of their unique and highly branched structures and a large number of terminal functional groups that show characteristics such as globular molecular shapes, high solubility, and the feasibility to be further modified through various chemical reactions of the peripheral groups [19]. The unique properties make them a prominent application for bioassay design. Here, unimolecular nanoparticles of crosslinked hyperbranched azo-polymer (HAP) decorated with Au colloids via covalent bond were used as a biosensing platform and have been demonstrated to anchor larger amounts of capture antibodies with high stability and bioactivity. The typical sandwich-type immunoassay procedure comprised the immunoreaction of capture antibody (goat polyclonal anti-SirT1, $Ab_1{}^{\#1}$) with target SirT1, followed by the attachment of detection antibody (rabbit polyclonal anti-SirT1, $Ab_1{}^{\#2}$), and {TiO_2–Au/Ab_2–HRP} labels, which featured secondary antibody horseradish peroxidase (Ab_2–HRP) linked to TiO_2–Au nanocomposites as electrochemical label. The SirT1 concentration, which was proportional to that of the Ab_2 linked to TiO_2–Au nanocomposites, could be readily examined through measurement of the electrochemical current. The proposed electrochemical measurement showed good performance in the monitoring of SirT1, which provides a promising approach to detect other significant proteins in the future.

Materials and methods

Chemicals and materials

N-Hydroxysuccinimide (NHS), 1-ethyl-3-3(3-dimethylaminopropyl) carbodiimide (EDC), dopamine, thionine, hydroxylamine hydrochloride, *N*-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), pentaerythritol tetrakis(3-mercaptopropionate) (PTMP), tetrakis (hydroxymethyl) phosphonium chloride (THPC), SirT1, goat anti-rabbit IgG-peroxidase (Ab_2–HRP), and bovine serum albumin (BSA) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). Goat polyclonal anti-SirT1 ($Ab_1{}^{\#1}$) and rabbit polyclonal anti-SirT1 ($Ab_1{}^{\#2}$) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Hyperbranched polymer was a gift from Liang Ding (Department of Chemistry, East China Normal University). TiO_2 (P-25, Φ = 30 nm) was purchased from Degussa. Chloroauric acid ($HAuCl_4$), trisodium citrate, and methanol were obtained from China National Medicines (China). Disodium hydrogen phosphate, potassium dihydrogen phosphate, and hydrogen peroxide (30% [w/v] solution) were purchased from Shanghai Chemical Reagent (China). Phosphate buffer solutions (PBSs) with different pH values were prepared by using Na_2HPO_4 and KH_2PO_4. Doubly distilled water was used throughout the experiments.

Apparatus and instruments

Scanning electron microscopy (SEM) was carried out on an S-4800 (Hitachi, Tokyo, Japan) equipped with an energy dispersive X-ray (EDX) analyzer. The transmission electron microscopy (TEM) images were performed by using a transmission electron microscope (JEOL model JEM 2100, Japan). The morphologies of the polymers were studied on a BioScope atomic force microscope (NanoScope IIIa SPM System, Digital Instruments, Veeco Instruments, USA). The element compositions of the products were measured by inductive coupled plasma–atomic emission spectroscopy (ICP–AES) on an iCAP 6300 (Thermo Fisher, Waltham, MA, USA). Light absorption was measured using an ultraviolet (UV)–visible spectrophotometer (Cary 50, Varian, USA). Electrochemical experiments were achieved on a CHI 660D electrochemical system (CH Instruments, China) with a conventional three-electrode system comprising a platinum wire as auxiliary electrode, a saturated calomel electrode (SCE) as reference electrode, and a bare or modified indium tin oxide (ITO) electrode as working electrode.

Synthesis of Au–HAP hybrids

Synthesis of unimolecular nanoparticle of crosslinked HAP

HAP (200 mg) and PTMP (12.2 mg) (1:2.5 M ratio) were dissolved in 250 ml of dried tetrahydrofuran (THF) at room temperature under nitrogen atmosphere. 1-Dodecylamine (1 mg, 10 wt%) was added as a catalyst, and the mixture was stirred for 2 h. The solution was concentrated under vacuum and precipitated into an excess of ethyl acetate, and the precipitate was isolated and dried under vacuum for 24 h to give the polymer as a solid.

Conjugation of Au colloids onto the unimolecular nanoparticle of crosslinked HAP

Gold colloid solution (1–2 nm in diameter) was prepared by the reduction of chloroauric acid with THPC as described by Duff and coworkers [20]. In a typical synthesis, 1 ml of the Au solution was diluted in 15 ml of doubly distilled water. Then, 10 ml of acetone dissolved in 0.5 mg of unimolecular nanoparticles of crosslinked HAP was added dropwise into the gold colloid solution under vigorous stirring; this process was conducted under the protection of a nitrogen atmosphere to minimize the oxidation of thiol groups. After 12 h of equilibration at room temperature, the solution mixture was centrifuged at 10,000 rpm for 30 min at room temperature and the supernatant solution was discarded. The sediments were then redispersed in doubly distilled water and centrifuged again. This purification cycle was repeated three times.

Preparation of TiO_2–Au nanocomposite

TiO_2–Au nanocomposites were prepared as follows. Commercial type Degussa P-25 TiO_2 composed of 70% anatase and 30% rutile was used in the fabrication experiments. TiO_2 solution was prepared by dispersing 3 mg of TiO_2 particles (P-25) in 35 ml of doubly distilled water and sonicated for 5 min. The TiO_2 solution was stirred with 1.88 ml of sodium citrate (0.1 mol L^{-1}) for 10 min to exchange hydroxyl ion with citrate anions [21]. Then, 0.125 ml of