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Graphene-based immunoassay for human lipocalin-2

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ABSTRACT

We have developed a highly sensitive immunoassay using graphene nano platelets (GNPs) for the rapid detection of human lipocalin-2 (LCN2) in plasma, serum, and whole blood. It has the dynamic range, linear range, limit of detection, and analytical sensitivity of 0.6 to 5120, 80 to 2560, 0.7, and 1 pg/ml, respectively. It is the most sensitive assay for the detection of LCN2, which has 80-fold higher analytical sensitivity and 3-fold lesser immunoassay duration than the commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kit. The functionalization of microtiter plate (MTP) with GNPs, dispersed in 3-aminopropyltriethoxysilane (APTES), provided the increased surface area that leads to higher immobilization density of capture antibodies. Moreover, the generation of free amino groups on MTP and GNPs by APTES enables the leach-proof covalent crosslinking of anti-human LCN2 capture antibody by its carboxyl groups using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) as the heterobifunctional crosslinker. The anti-LCN2 antibody-bound MTPs were highly stable given that they did not show any significant decrease in their functional activity when stored at 4 °C in 0.1 M phosphate-buffered saline (PBS) for 8 weeks. The developed immunoassay correlated well with the conventional ELISA, thereby demonstrating its high precision and potential utility for highly sensitive analyte detection in industrial and clinical settings.

Graphene has been the most widely used nanomaterial during the past decade for a plethora of highly diversified applications [1–6]. It is a two-dimensional planar sheet of sp^2-bonded carbon atoms, which are packed densely in a honeycomb crystal lattice. It has been employed extensively in biosensors due to its low cost, high electrical conductivity, absence of metallic impurities, high thermal conductivity, high mechanical strength, and larger surface area. The effective surface area of graphene (2630 m^2/g) has been found to be 2-fold higher than that of carbon nanotubes [7].

Lipocalin-2 (LCN2[1] [8], also known as neutrophil gelatinase-associated lipocalin, NGAL), is a small 25-kDa secretory glycoprotein that is found in neutrophils and certain epithelia such as renal tubules. It is an important prognostic and diagnostic biomarker for several medical conditions. The LCN2 threshold in healthy humans is 0.7 ng/ml, but there are many diseases and disorders that induce an up-regulation of LCN2 based on the pathophysiology. LCN2 expression is dramatically increased in ischemic [9] or nephrotoxic [10] injury. It is an early and sensitive biomarker for acute renal injury [11–14] because its levels rise in urine and blood within just a few hours. Higher levels of LCN2 may lead to acute renal failure. It is also relevant in cases of cardiovascular surgery; coronary heart disease [15,16]; kidney transplants [17–19]; bacterial infections [20,21]; fatty liver disease; obesity and its associated pathologies; several types of cancers such as breast [22], pancreatic [23], ovarian [24], and esophageal [25]; and obesity-induced metabolic disorders [26] such as insulin resistance, type 2 diabetes mellitus, and cardiovascular disorders. The prognosis of acute renal failure is usually done by creatinine or cystatin C measurements, but they respond only after a few days of the renal function deterioration. The down-regulation of LCN2 is also a diagnostic marker for many diseases such as glioblastoma [27], where LCN2 is down-regulated as a subsidiary mechanism of enhancing chemoresistance in glioblastoma cells. The LCN2 threshold is also down-regulated in ovarian cancer [24] and microglial cells [28]. Studies have also suggested that LCN2 is a predictive marker for excessive exposure to high electromagnetic fields that significantly decreases the body threshold of LCN2 [29].

The recent commercial availability of several sandwich enzyme-linked immunosorbent assay (ELISA) kits, which determine the pathophysiological LCN2 levels in biological samples, provides the highly useful information to physicians for the

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[1] *Abbreviations used:* LCN2, lipocalin-2 (also known as neutrophil gelatinase-associated lipocalin, NGAL); ELISA, enzyme-linked immunosorbent assay; GNP, graphene nano platelet; EDC, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride; PBS, phosphate-buffered saline; Mes, 2-(*N*-morpholino)ethanesulfonic acid; TMB, 3,3′,5,5′-tetramethylbenzidine; BSA, bovine serum albumin; KOH, potassium hydroxide; APTES, 3-aminopropyltriethoxysilane; SA–HRP, streptavidin-conjugated horseradish peroxidase; PBST, PBS with 0.05% Tween 20; UPW, ultrapure water; MTP, microtiter plate; LOD, limit of detection.

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effective management of various renal malfunctions and cancers. The developed graphene nano platelet (GNP)-based sandwich ELISA procedure (see Fig. 1 for schematic of procedure) is superior to our previous developed covalent sandwich ELISA [30] and the conventional ELISA being used in commercially available kits in terms of higher sensitivity and reduced immunoassay duration. The assay components in the commercially available human LCN2 sandwich ELISA kit from R&D Systems (USA) were employed for the developed and conventional ELISAs, which were performed under the same conditions and at the same time. The developed GNP-based sandwich ELISA correlated well with the conventional ELISA for the detection of various concentrations of LCN2 spiked in diluted human plasma. Therefore, it will be a highly prospective format for the development of rapid and highly sensitive in vitro diagnostic kits and immunoassays for various disease biomarkers and analytes.

Materials and methods

Materials

1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), phosphate-buffered saline (PBS, 0.1 M, pH 7.4), 2-(*N*-morpholino)ethanesulfonic acid (Mes, pH 4.7), a 3,3′,5,5′-tetramethylbenzidine (TMB) substrate kit, and bovine serum albumin (BSA) were purchased from Thermo Scientific (USA). Potassium hydroxide (KOH) and 3-aminopropyltriethoxysilane (APTES) were obtained from Sigma–Aldrich. GNPs (diameter = 5 μm) were purchased from Cheap Tubes (USA). The human LCN2 DuoSet kit, containing all sandwich ELISA components (i.e., anti-human LCN2 capture antibody, biotinylated anti-human LCN2 detection antibody, and streptavidin-conjugated horseradish peroxidase, SA–HRP), was procured from R&D Systems. PBS (0.1 M, pH 7.4) with 1% (v/v) BSA was used for reconstituting all assay components, PBST (PBS with 0.05% Tween 20) was used as washing buffer, and ultrapure water (UPW, 18 MΩ, Direct Q, Millipore, USA) was used for preparing buffers, KOH, and APTES. EDC was reconstituted in 0.1 M Mes (pH 4.7). The human whole blood, serum, and plasma were purchased from Streck (USA), HyTest (Finland), and Biological Specialty (USA), respectively. The human LCN2-spiked samples were prepared by mixing various concentrations of human LCN2 in 1:1000 diluted human plasma, serum, and whole blood. In addition, 1 mg of GNPs was mixed with 1 ml of 0.25% APTES and dispersed by keeping in an ultrasonic bath for 1 h before use in the developed immunoassay. The dilutions of human LCN2 were made in BSA-preblocked sample vials in order to minimize the analyte loss due to nonspecific binding on sample tube surfaces [31]. The assay temperature was maintained at 37 °C using a thermostat obtained from Labnet International (USA), and the absorbance was measured by a Tecan Infinite M200 Pro microplate reader from Tecan (Austria).

Polystyrene, poly(methyl methacrylate), and Zeonex were procured from Microfluidic Chip Shop (Germany), polycarbonate and cellulose acetate were procured from VTT (Finland), and Zeonor was purchased from Zeon Chemicals (Germany). The pressure-sensitive adhesive and bottomless 96-well microtiter plate (MTP) were bought from Adhesive Research (Ireland) and Greiner Labortechnik (Germany), respectively.

GNP functionalization of MTP and immobilization of capture antibody

Each MTP well was treated with 100 μl of 1.0% (w/v) KOH for 5 min, followed by five washings with 300 μl of UPW. It was then functionalized with GNPs by incubating with GNPs (1 mg/ml) in 0.25% APTES for 1 h inside the fume cabinet, followed by five washings with 300 μl of UPW. EDC-activated anti-human LCN2 antibody was made by incubating 990 μl of 2 μg/ml anti-human LCN2 antibody with 10 μl of EDC (4 mg/ml) for 15 min at 37 °C. It was then added to each of the GNP-functionalized wells (100 μl), incubated for 1 h at 37 °C, and subsequently washed six times with 400 μl of PBST.

Sandwich immunoassay procedure

The nonspecific binding sites on anti-human LCN2 antibody-bound MTP were blocked by treating with 1% (v/v) BSA for 30 min at 37 °C, followed by six washes with 400 μl of PBST. Subsequently, 100 μl of each of the varying concentrations of human LCN2 (0.6–5120 pg/ml) was incubated in the anti-human LCN2-coated MTP for 1 h at 37 °C, followed by six washes with 400 μl of PBST. Thereafter, 100 μl of biotinylated anti-human LCN2 detection antibody (100 ng/ml) was added, incubated for 1 h at 37 °C, and then washed six times with 400 μl of PBST. This was followed by adding 100 μl of SA–HRP (diluted 1:200) to each MTP well, incubating for 20 min at 37 °C, and washing six times with 400 μl of PBST. Subsequently, the TMB substrate was added (as per the manufacturer's guidelines) and the enzyme-substrate reaction was stopped after 20 min by adding 50 μl of 2 N H_2SO_4. The absorbance was measured at 450 nm with reference at 540 nm. All of the experiments were done in triplicate with 0 ng/ml human LCN2 (in 0.1 M PBS, pH 7.4, with 1% BSA) as control, whose absorbance was subtracted from all of the assay values. The conventional sandwich ELISA procedure was followed as per the manufacturer's guidelines provided in the product information sheet without any modification. The developed ELISA without using GNPs was also performed for comparison. Various experimental process controls were also employed in order to check the efficiency of BSA blocking; the nonspecific interactions of BSA with LCN2, biotinylated anti-LCN2 antibody, and SA–HRP; and the nonspecific interaction of capture anti-LCN2 antibody with biotinylated anti-LCN2 antibody. All datasets obtained from the developed and conventional human LCN2 sandwich ELISAs were subjected to standard curve analysis using SigmaPlot software (version 11.2). The EC_{50}, R^2, and hill slope values were determined from the report generated by the software during standard curve analysis based on the four-parameter logistic function. The analytical sensitivity, limit of detection (LOD), and intra- and interday variability were determined by the standard procedures as specified in our previous reports [32,33]. Briefly, the LOD and analytical sensitivity were calculated by:

$$O.D._{\cdot\mathrm{LOD}} = \text{Average } O.D._{\cdot\mathrm{Blank}} - 3\left(S.D._{\cdot\text{Min. Analyte Conc.}}\right) \quad (1)$$

$$O.D._{\cdot\mathrm{AS}} = \text{Average } O.D._{\cdot\mathrm{Blank}} - 3(S.D._{\cdot\mathrm{Blank}}), \quad (2)$$

where $O.D._{\cdot\mathrm{LOD}}$ and $O.D._{\cdot\mathrm{AS}}$ are the optical densities corresponding to LOD and analytical sensitivity, respectively; $O.D._{\cdot\mathrm{Blank}}$ is the optical density of the blank; and $S.D._{\cdot\text{Min. Analyte Conc.}}$ and $S.D._{\cdot\mathrm{Blank}}$ are the standard deviations of the minimum analyte concentration and blank, respectively.

Results and discussion

LCN2 is an early and sensitive biomarker for acute renal injury [34–37] that increases in urine and blood within just a few hours. The higher levels of LCN2 may lead to acute renal failure. The LCN2 levels are also increased in patients with colitis, procitis [38], contrast-induced nephropathy [39], β-thalassemia [40], coronary heart disease [41], and heart failure [42]. It is up-regulated in anemia [43], inflammation, and cancer [44,45]. In addition, it is an early screening biomarker for ovarian cancer [24] and enables the early prediction of urinary tract infections [46]. Therefore,