PHOTON-UPCONVERTING NANOPARTICLES AS A NOVEL BACKGROUND-FREE LABEL IN IMMUNOASSAYS

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Abstract

Photon-upconverting nanoparticles (UCNPs) have become an attractive label in immunoassays because their anti-Stokes luminescence can be excited by the NIR laser and detected in the VIS region without optical background interference. Further advantages of UCNPs include good photostability, large anti-Stokes shifts, and multiple narrow emission bands that can be used for multiplexed detection. We have developed a competitive upconversion-linked immunosorbent assay (ULISA) for detection of the pharmaceutical diclofenac (DCF) in surface waters. Silica-coated UCNPs (50 nm in diameter) with carboxyl groups on the surface were synthesized and conjugated with the secondary anti-IgG antibody. The structure and monodispersity of the nanoconjugates was studied by TEM and agarose gel electrophoresis. Using a highly affine anti-DCF primary antibody, the optimized ULISA provided a detection limit of 50 pg·mL^{-1}.

Keywords: Photon-upconversion nanoparticle, immunoassay, bioconjugation, antibody, diclofenac

1. INTRODUCTION

Photon-upconverting nanoparticles (UCNPs) are lanthanide-doped nanocrystals that show anti-Stokes emission. The energy transfer up-conversion is a nonlinear optical process characterized by the absorption of two or more photons and leading to the emission of a single photon at a shorter wavelength [1]. Upconversion has been known since the 1960s [2]; however, the applications were limited to bulk glass or crystalline materials [3]. An inorganic upconversion label contains a crystalline host and a dopant (typically lanthanide ions) added at a low concentration. The dopant provides luminescent centers, and the host lattice with its crystal structure ensures a matrix to bring these centers into optimal positions [4].

The recent progress in nanotechnology has evoked an increasing interest in the development of synthesis procedures, facilitating the preparation of highly efficient, small UCNPs with a narrow size distribution that can form transparent dispersions in various solvents [5]. Unlike commonly used luminescent biological labels (such as organic dyes and quantum dots), UCNPs are advantageous in many aspects; they are characterized by a practically zero autofluorescence background, large anti-Stokes shifts allowing easy separation of the excitation and detection channels, multiple and narrow emission bands tunable individually for the multiplexed detection of analytes [6]. The high photostability enables the use of very intense excitation power densities without photobleaching, which makes possible to detect a few or even a single UCNP [7].

The unique photophysical properties of UCNPs make them suitable as reporters in optical biosensors and biomolecular binding assays [8]. The potential of UCNPs in diagnostics was first demonstrated by Tanke et al. in 1999 [9]. Since then, several heterogeneous upconversion-linked immunosorbent assays (ULISAs) have been reported e.g. for the detection of cardiac troponin I [10], prostate specific antigen [11], or human chorionic gonadotropin [12]. The UCNPs were also used in homogeneous competitive immunoassays for the detection of small molecules such as estradiol [13] and folate [14]. Here, we present preparation of monodisperse and

stable upconversion reporters and their application for the detection of DCF in water samples; the contribution is in part based on our previous publication [15].

2. MATERIAL AND METHODS

2.1. Chemicals and Reagents

All standard chemicals and DCF sodium salt were obtained from Sigma-Aldrich (Germany). Carboxyethylsilanetriol sodium salt was obtained from ABCR GmbH (Germany). Horse anti-mouse IgG was purchased from Vector Laboratories (USA). The monoclonal anti-DCF antibody 12G5 was generated in mice using a DCF-thyroglobulin conjugate as described previously [16].

2.2. Synthesis of antibody-modified UCNPs

The UCNPs of 42.5 ± 4.9 nm in diameter were synthesized by high-temperature coprecipitation [17] and coated by carboxyl-silica layer using reverse microemulsion method [18]. For the bioconjugation reaction, COOH-UCNPs were first activated by 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS) for 30 min [15]. A dispersion of 100 µL of activated COOH-UCNPs (1 mg·mL^{-1}) was mixed with 100 µL of horse anti-mouse IgG in 100 mM sodium borate buffer, pH 9.0. Different IgG concentrations were employed and incubated for 90 min at room temperature: 0.1; 0.02; 0.01 and 0.002 µg·mL^{-1}. The bioconjugates were centrifuged for 10 min at 4 000 g, dispersed in UCNP assay buffer (50 mM Tris, 150 mM NaCl, 0.05 % NaN_3, 0.01 % Tween 20, 0.05 % bovine γ-globulin (BGG), 0.5 % bovine serum albumin (BSA), 0.2 % poly(vinyl alcohol) 6000 (PVA), pH 7.75) and sonicated for 5 min.

2.3. Characterization of UCNPs

Transmission electron microscopy (TEM) was performed on a Tecnai F20 (FEI, Netherlands). About 4 µL of UCNPs were deposited on a 400-mesh copper EM grid coated with a continuous carbon layer and negatively stained with 2 % (w/v) aqueous solution of uranyl acetate to increase the contrast of the silica shell. The hydrodynamic diameter and zeta potential of UCNP suspensions were determined on a Zetasizer Nano SZ (Malvern, U.K.).

Agarose gel electrophoresis was used to further characterize the COOH-UCNPs and their bioconjugates; the electrophoresis conditions were 0.5 % w/v agarose, 45 mM Tris, 45 mM H_3BO_3 with pH 8.6, 15 min at 100 V. The UCNP samples were mixed in a ratio of 10:1 with 50 % w/w glycerol and 8 µL aliquots were loaded onto the gel. A custom-built upconversion reader (Chameleon multilabel microplate reader, Hidex, Finland) equipped with 4 W continuous 980 nm laser was used to scan agarose gels with a spatial resolution of 0.5 mm [19].

2.4. Upconversion-Linked Immunosorbent Assay

High-binding 96-well microtiter plate (Corning, Germany) was coated by the BSA-diclofenac conjugate (concentration 1 µg·mL^{-1}) in 50 mM $NaHCO_3$/Na_2CO_3, 0.05 % NaN_3, pH 9.6 over night at 4 °C. All subsequent steps were carried out at room temperature. After each step, the plate was washed four times with 250 µL of washing buffer (50 mM NaH_2PO_4/Na_2HPO_4, 0.01 % Tween 20, 0.05 % NaN_3, pH 7.4). The free binding sites were deactivated using 250 µL of 1 % BSA in 50 mM NaH_2PO_4/Na_2HPO_4, 0.05 % NaN_3, pH 7.4 for 1 h. The standard solutions of DCF in PBS (100 µL per well) were added followed by 100 µL of mouse monoclonal IgG 12G5 (0.5 µg·mL^{-1}) and incubation for 60 min. Afterwards, the plate was incubated with UCNP-Ab conjugate (concentration 10 µg·mL^{-1} of UCNP cores) for 60 min and the upconversion luminescence was read out from dry empty wells using an upconversion reader [19]. Each well was scanned 100 times with step 0.4 mm in a raster shape (10×10) with integration times of 500 ms. Truncated average was calculated for each well after

discarding the 10 highest and 10 lowest measurements to account for local irregularities on the microtiter well surface that result in signal outliers.

3. RESULTS AND DISCUSSION

Oleic acid-capped UCNPs were coated with a silica shell exposing carboxylic acid functional groups on the surface. The carboxyl groups improve the dispersibility in water and serve as attachment sites for subsequent conjugation steps. The diameter of COOH-UCNPs was determined by transmission electron microscopy (TEM, 46.9 ± 5.0 nm; **Figure 1A**) and atomic force microscopy (AFM, 45.4 ± 7.6 nm). The COOH-UCNPs were conjugated to a secondary anti-IgG antibody via standard EDC/sulfo-NHS chemistry. A low concentration of COOH-UCNPs was utilized to prevent that one antibody molecule binds to several UCNPs, which would lead to aggregation. The conjugates were characterized by agarose gel electrophoresis (**Figure 1B**), dynamic light scattering (DLS), zeta potential measurements, and FT-IR spectroscopy. The lowest degree of aggregation was observed when the concentration of COOH-UCNPs in the reaction mixture was 1 $mg \cdot mL^{-1}$. The conjugation of the secondary antibody reduced the negative surface potential of the COOH-UCNPs as shown by zeta potential measurements and led to a stronger retardation in the agarose gel. The IgG-UCNP conjugates were purified from an excess of unbound secondary anti-mouse IgG and components of the reaction mixture by differential centrifugation. At first, the bioconjugates were centrifuged at 10 000 g, which led to strong nanoparticle aggregation. Afterwards, the centrifugation speed was reduced to 4 000 g followed by 5 min of sonication, which provided monodisperse purified IgG-UCNP.

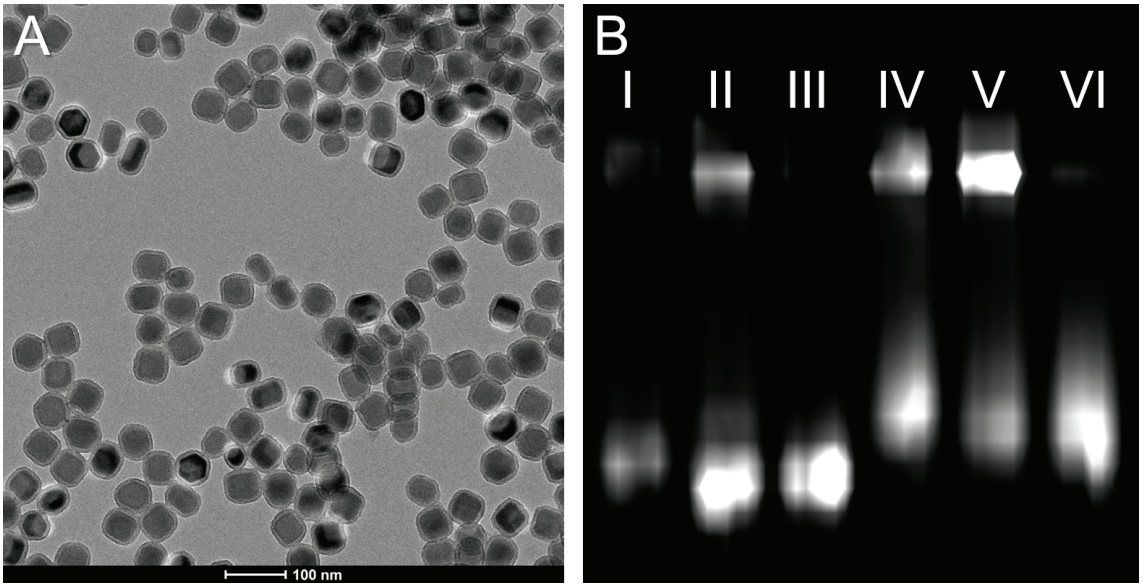

Figure 1 A) TEM image of silica-coated UCNPs exposing carboxyl groups on the surface. B) Characterization of UCNP-Ab conjugates by agarose gel electrophoresis. The unmodified COOH-UCNPs and IgG-UCNP conjugates (I and IV) were centrifuged at 4 000 g (II and V) and sonicated (III and VI)

The synthesized and characterized UCNP-Ab conjugates were employed as a detection element in competitive ULISA for the detection of DCF. Generally, a low concentration of coating antigen ensures that the free analyte can compete efficiently for the binding sites of the detection antibodies. On the other hand, the signal generation has to be strong enough for a reliable readout. Two different coating conjugates were prepared consisting of BSA-DCF. The conjugates were analyzed by MALDI-TOF mass spectrometry, which showed a coupling density of either 5.7 or 10 DCF residues per BSA molecule. When the conjugate with the higher degree of derivatization was used, the signals were about twice as high but also showed stronger signal fluctuation and a hook effect, which may be the consequence of two binding sites of IgG molecules forming

cyclic complexes. By contrast, the conjugate exposing 5.7 DCF residues per BSA molecule yielded more stable signals, slightly lower IC_{50} value and a lower detection limit for DCF. Consequently, this coating conjugate was used in further experiments. An optimal signal generation was observed with a coating concentration of 1 µg·mL^{-1}.

The competition step including free DCF and anti-DCF detection antibody was performed in analogy to a conventional ELISA [16] with the enzyme-mediated color generation replaced by IgG-UCNP conjugates as a direct luminescent reporter. The degree of UCNP surface substitution did not significantly affect the IC_{50} or the LOD, the concentration of 10 µg·mL^{-1} of IgG-UCNPs resulted in the most reproducible upconversion signal generation. In contrast to the UCNP-bound secondary antibody, the primary anti-DCF antibody is directly involved in the competition step and both the upconversion signal intensity and the IC_{50}/LOD for DCF were strongly affected by the antibody concentration. A higher concentration leads to a higher signal intensity because more antibodies can bind to the DCF-BSA coating conjugate, but they also consume a larger amount of free DCF and thus deteriorate the assay sensitivity. A concentration of 0.25 µg·mL^{-1} primary anti-DCF antibody yielded an optimal balance between signal generation and sensitivity for the determination of DCF and was used in further experiments. The optimized ULISA assay (**Figure 2**) provided LOD of 50 pg·mL^{-1} and IC_{50} of 0.23 ng·mL^{-1}. The LOD of the ULISA was slightly higher than the value of a conventional ELISA (7.8 pg·mL^{-1}) [16], however the ULISA allows for a faster and easier signal generation within 140 min [15]. The assay time can be improved even further by using a direct assay scheme based on competition of free DCF with a tracer consisting of DCF, BGG and UCNP [20]. In this case, the BGG was employed (1) to minimize steric hindrance between the anti-DCF antibody and the UCNP surface and (2) to prevent non-specific adsorption of the tracer to the microtiter plate. The assay provided a comparable LOD of 20 pg mL^{-1} with total analysis time reduced to 70 min.

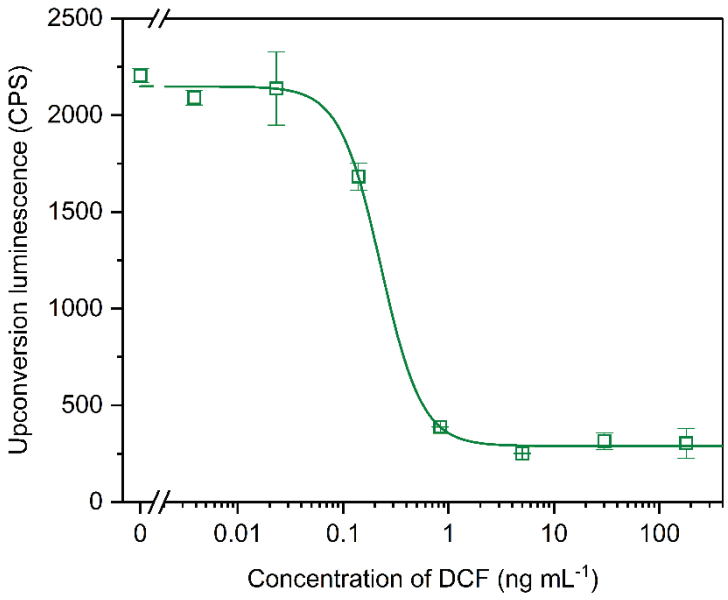

Figure 2 Calibration curve of optimized ULISA for the detection of DCF (LOD 50 pg·mL^{-1}; IC_{50} 0.23 ng·mL^{-1}). Error bars represent standard deviations of three replicate wells

For the analysis of real samples, drinking water (from Munich) and two surface water samples (lake Wörthsee and river Würm) were collected in Southern Bavaria (Germany). The DCF concentrations were, however, too low to be detectable in the unspiked water samples, therefore, each sample was additionally spiked with either 1 or 10 ng·mL^{-1} of DCF. The spiked samples were typically diluted at least by a factor of 3 prior to the immunoassay to reduce the matrix effects. The DCF concentrations in real samples determined by ULISA are shown in **Table 1**. The deviations from the spiked concentrations were observed which might be caused by

the effect of sample matrix on the binding of the nanoparticle luminescent label, the metal ion can disrupt non-covalent interactions by shielding charges and destabilizing hydrogen bonds.

Table 1 Detection of DCF in unspiked and spiked real water samples using ULISA

Sample	Spiked concentration [ng·mL^{-1}]	ULISA [ng·mL^{-1}]
Munich tap water	0	< LOD
	1	1.53 ± 0.28
	10	15.0 ± 5.4
Lake Wörthsee	0	< LOD
	1	1.81 ± 0.06
	10	10.2 ± 3.8
River Würm	0	< LOD
	1	1.05 ± 0.26
	10	10.9 ± 1.6

4. CONCLUSION

A heterogeneous upconversion-linked immunosorbent assay was developed for the detection of non-steroidal anti-inflammatory drug diclofenac. Silica-coated UCNPs ($NaYF_4$ doped with Yb^{3+} and Er^{3+}, 50 nm in diameter) exposing carboxyl groups on the surface were synthesized and successfully conjugated to a secondary anti-IgG antibody. The structure and monodispersity of the nanoconjugates was studied by TEM and agarose gel electrophoresis. Using a highly affine anti-DCF primary antibody, the optimized ULISA assay reached the LOD of 50 pg·mL^{-1}. This performance comes close to a conventional ELISA without the need for an enzyme-catalyzed signal amplification step. The ULISA was further used for the analysis of drinking and surface water samples, the achieved results were consistent with ELISA.

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