Novel fluorescent nanoparticles for ultrasensitive identification of nucleic acids by optical methods

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ABSTRACT

For decades, the detection of nucleic acids and their interactions at low abundances has been a challenging task. Present nucleic acid diagnostics are primarily based on enzymatic reactions including sequencing, polymerase-chain reaction and microarrays. However, the use of enzymatic amplification interferes with the initial biomolecular system, is limited to in vitro assays, often time consuming and rather expensive. Therefore, there is interest in new amplification-free detection methods. A tremendous progress has been made in fluorescence based optical detection of biomolecules. In this work, we aimed at developing efficient tools for amplification-free nucleic acid detection. The result of simple and inexpensive polymerization in the presence of fluorescent dyes and additional functionalization reagents was ultra-bright fluorescent nanoparticles modified with additional groups for bioconjugation.

Keywords: nucleic acid, fluorescence, nanoparticle, butyl acrylate, radical polymerization.

1 INTRODUCTION

Today, nucleic acid diagnostics uses enzymatic target amplification in order to achieve the required sensitivity and specificity. This includes polymerase-chain reaction (PCR), sequencing and microarrays [1,2]. Over the past decade, there has been a growing concern on the accuracy of these methods. PCR in particular manipulates the initial biomolecular system, resulting in a high risk of affecting the target stoichiometry [3]. Moreover, not all the sequences can be amplified.

Alternative methods to enzyme-assisted assays include optical and electrochemical amplification-free techniques [3]. For these detection methods, fluorescence is often being applied. Fluorescence is inexpensive and robust, which makes it useful for life science research, nanotechnology and clinical assays [2]. However, it has so far not been possible to develop an amplification-free strategy with the required sensitivity, despite great progress in the field [3]. In this work, we aimed at developing new strategies for preparation of ultra-bright fluorescent nanoparticles (NPs) and to demonstrate their potential in amplification-free nucleic acid detection. For this, fluorophores have to be bright, photostable and nontoxic [4]. As to polymerization, several approaches have been developed. Among them, polymerization of styrene by nucleated polymerization in the presence of seed material. The styrene is first undergoing nucleation to form small particles that are further polymerized by seeded polymerization [5,6,7]. Other successful approaches include monodisperse silica NPs, quantum dot (QD) NPs and gold NPs [8].

In this work, our major goal was to find a simple and inexpensive way of making ultra-bright fluorescent NPs useable for the amplification-free detection of nucleic acids. This means that the nanoparticles have to be less than 50 nm in diameter and monodisperse. In addition, the particles need to contain additional functionality for the attachment to biomolecules. Herein we describe how these challenges have been addressed by the radical polymerization of butyl acrylate in the presence of additional molecules and selective click chemistry (Scheme 1, Figure 1).

2 EXPERIMENTAL

All chemicals were obtained from Sigma-Aldrich and used as received. The cyanine 5 dye and TBTA were obtained from Lumiprobe LLC. Stock solutions for click chemistry were prepared as described in the Lumiprobe protocol for click chemistry labeling of oligunucleotides and DNA. The miRNA-21 probe precursor was obtained from IDT.

2.1 Butyl acrylate polymerization

Polymerization of BA was made with a corresponding dye and 3-azidopropan-1-amine by adding 0.0076 g of sodium bicarbonate (90.5 µmol) to 30 mL MQ water in a round bottom flask under continuously stirring. Afterwards, 0.065 g of sodium dodecyl sulfate (225.4 µmol) was added to the solution followed by 5.6 mL of aqueous BA (38.9 mmol). Hereafter, the dye and 2 µL of 3-azidopropan-1-amine (20.4 nmol) were added to the reaction. The reaction mixture were deaerated and heated to 60 ºC, before initiation with 0.3426 g of AP (1.5 mmol) in 1 mL MQ water and left to react under heat for 30 min. Then, the

reaction mixture was quenched with 0.330 g hydroquinone (3 mmol) in 2 mL methanol:water (1:1) and afterwards cooled down to room temperature. The solvent was evaporated on a rotary vacuum evaporator, washed with methanol two times. Finally the NPs were resuspended in acetone:methanol (1:4) in total volume of 50 mL.

Dyes used were cyanine 5, perylene, AY, and TO. In all cases 0.005 g (0.005%) of the dye was added to the reaction, except for TO (0.0025 g (0.0025%) was added). The reaction mixture was protected from light to prevent the bleaching of the dyes [9].

2.2 Click chemistry

CuAAC "click" reaction was performed for the azide functionalized perylene-NPs with the alkyne functionalized oligonucleotide (ON) (table 1). The azide-perylene NPs (in excess) 3.8 µM /38 µM and ON (10 nmol in MQ water) was mixed with 20 µL triethylammonium acetate buffer (1 M), $CuSO_4$ (200 µM), and ascorbic acid (20 mM) in total volume of 200 µL, 50% MQ water and 50% DMSO. The reaction mixture was deaerated, left at room temperature for 48 hours under shaking. The products from the click reaktion were analyzed on a 2.5 % agarose gel.

Sequence	*Mw (g/mol)*
5'- /Hexynyl/GCA TGA AGG GCC TCG G-3'	5107.4

Table 1. Oligo ribonucleotide sequence

2.3 Imaging of the nanoparticles

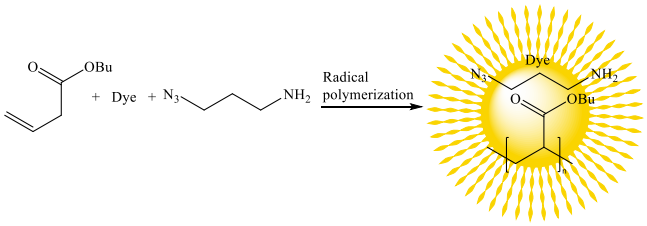
The NP characterization was carried out by dynamic light scattering (DLS) and transmission electron microscopy (TEM) at Aarhus University, Denmark. The TEM analysis was made on a FEI Tecnai G2 Spirit with a LAB6 filament operated at 120keV and a bottom mounted TemCam-F416® direct detector (CMOS) obtained the images. The DLS analysis was made on samples that were diluted 1:50 in water using a Zetasizer Nano Z system (Malvern).

2.4 Fluorometry

Fluorescence was measured using a PerkinElmer LS 55 luminescence spectrometer equipped with a Peltier temperature controller. The limit of detection (LOD) was measured at rt in 1xPBS, pH 7.2, by serial dilution of each sample. The experiment values are average of two independent measurements with deviation within 130 A.U. The titration was done in the concentrations 1×10^{-6} µM, 1.87×10^{-3} µM, 3.74×10^{-2} µM, 5 µM, 50 µM and 100 µM.

3 RESULT AND DISCUSSION

First, we prepared a series of novel NPs by a simple radical emulsion polymerization of butyl acrylate (BA) in the presence of different fluorescence dyes and 3-azidopropan-1-amine (scheme 1) [10,11]. From previously described approaches, it is known that the water-soluble ammonium persulfate (AP) by fare benefits the polymerization rate and efficiency compared to the oil-soluble 2,2'-azobisizobutyronitrile (AIBN) [10,11]. Moreover, the NPs made by AIBN seem to be less monodisperse and often too great of size to be applied in bio imaging [10].



Scheme 1. Synthesis of functionalized fluorescent NPs by radical polymerization of BA.

The polymerization was preceded with perylene, cyanine 5, acridine yellow (AY) and thiazole orange (TO) dyes (figure 1). The reaction was carried out for 30 minutes in an inert system and afterwards quenched with hydroquinone as in the literature [10].

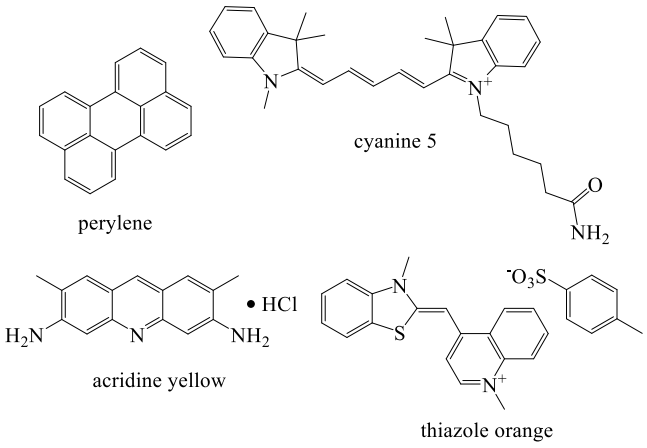


Figure 1. Dyes used for polymerization of fluorescence NPs.

The nanoparticles made by BA polymerization were initially characterized by DLS and TEM. DLS and TEM are frequently used to determine the size and dispersity of particles in the nanoscale [12]. DLS is an ensemble technique that exploits scattered light from nanoparticles to determine their average approximated hydrodynamic size [12,13]. In turn, TEM is a direct imaging method that enables measurements of each individual particle diameter [12].

In our work we aimed at coupling the NPs to a target oligonucleotide sequence. As a proof of principle, we

selected a complementary sequence to miRNA-21, which is known as a cancer development biomarker [7,14]. For mild bioconjugation strategies, click chemistry is a great technique [15,16]. Copper-catalyzed azide-alkyne cycloaddition (CuAAC) in particular is proven to be effective and specific for oligonucleotide functionalization [16].

3.1 Imaging

The NPs obtained by the synthesis were characterized by TEM and DLS. This showed promising results on especially perylene NPs showing small and monodisperse NPs (figure 2 and 3).

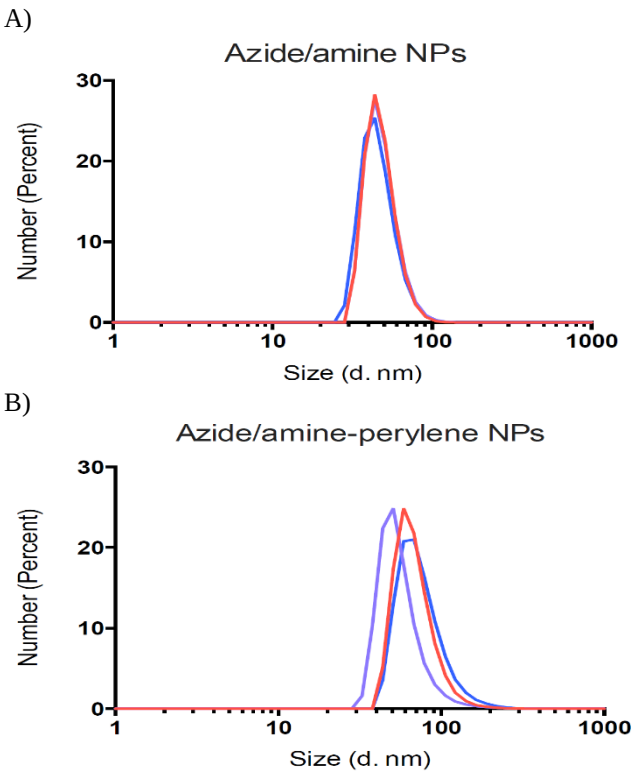


Figure 2. DLS results. Size distribution by number of A) azide/ amine NPs and B) azide/ amine-perylene NPs

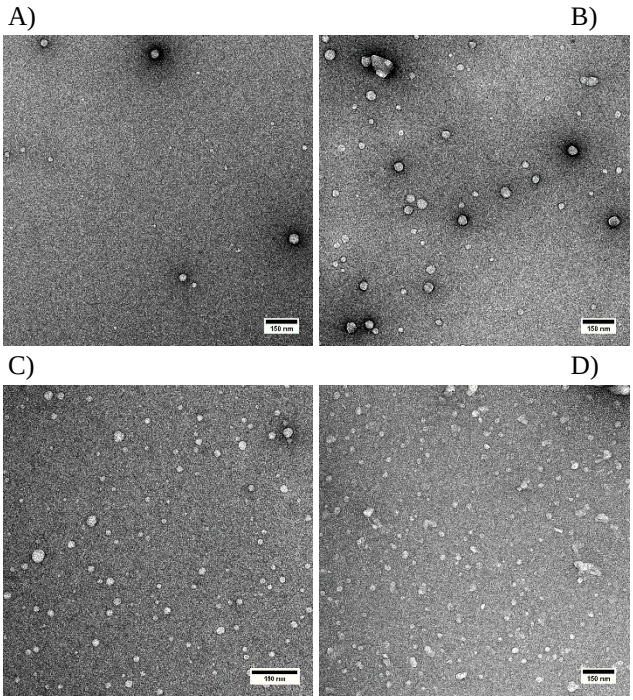


Figure 3. Zoomed TEM images, scale bar of 150 nm, of A) dye-free NPs, B) perylene NPs, C) azide dye-free NPs and D) azide perylene NPs.

DLS measurements show mean diameter sizes of 33.9 nm and 35.9 nm and polydispersity index (PDI) of 0.18 and 0.21 respectively for non-fluorescent NPs and perylene NPs. For cyanine 5 NPs, the mean diameter was considerably higher with a mean diameter of 247,7 nm and it was more polydisperse as well. We did not manage to determine the mean size for AY NPs on DLS. We determined the average diameter by TEM of non-fluorescent NPs (15.3 ± 10.1 nm), perylene NPs (26.6 ± 12.0 nm), cyanine 5 NPs (13.2 ± 6.2 nm) and AY NPs (8.8 ± 4.3 nm).

The azide/ amine-non-fluorescent NPs and the azide/ amine-perylene NPs showed mean diameters of 47,6 nm and 67.52 nm, respectively, whereas the azide/ amine-AY NPs display mean diameter of 100,6 nm by DLS measurements. Noteworthy was that the azide/ amine-non-fluorescent NPs and the azide/amine-perylene NPs showed low hoterogeneity with PDI of 0,20 and 0,43 respectively where the azide/ amine-AY NPs heterogeneity were higher PDI of 0,49.

3.2 Click chemistry

The products were analysed by agarose gel electrophoresis (Fig. 4). The gel confirms a full conversion of the initial ON to product conjugate.

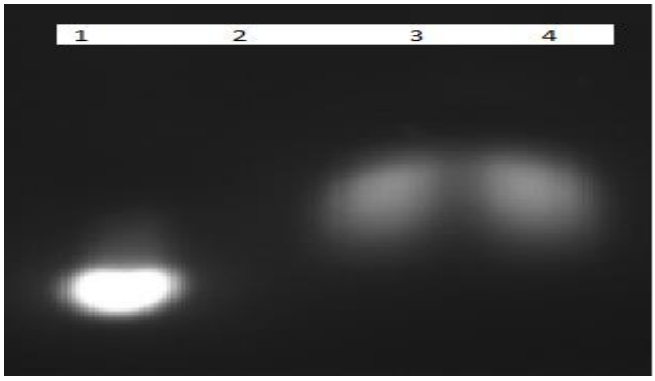


Figure 4. 2.5 % agarose gel of NP-oligonucleotide. ON (1), azide/ amine perylene-NPs (2), click product ON-perylene-NPs, (3.8 µM azide/ amine-perylene NP) (3), click product ON-perylene-NPs, (38 µM of azide/ amine-perylene NP) (4).

3.3 Fluorometry

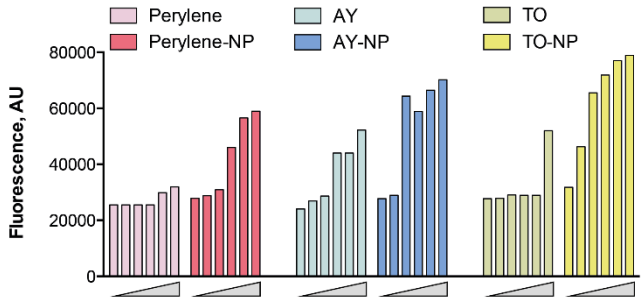


Figure 5. Fluorometry results of the titration and LOD determination for the monomolecular dye controls and the azide-fluorescent nanoparticles.

The limit of detection (LOD) for the NPs were respectively 2 nM, 0.5 µM and 5 µM for the perylene NPs, TO NPs and the AY NPs. The observed LOD for the precursor monomolecular dyes was 150 µM, 70 µM and 30 µM for the perylene, TO and AY respectively.

4 CONCLUSION

Herein we synthesized and tested a panel of novel fluorescent nanoparticles. We prove the possibility to make homogeneous nanoparticles in a size range usable for nucleic acid detection by optical methods. The most promising approach according to the DLS and TEM analysis was with perylene, due to small sized monodisperse NPs. Furthermore, we have developed a method for functionalizing the NPs with miRNA-21 targeting oligonucleotide probe by convinient click chemistry approach. The limits of target detection were advantageous for the NPs when compared to monomolecular organic dyes. Alltogether, this work demonstrates how advances in organic synthesis can benefit synthetic biology of nucleic acids and aid the development of novel effective diagnostic methods.

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