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From particle to platelet: Optimization of a stable, high brightness fluorescent nanoparticle based cell detection platform

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Abstract

Nanoparticles are increasingly used as diagnostic tools due to the ease with which their surface chemistry, optical and physical properties can be controlled. Molecules, drugs, enzymes and fluorophores can be protected within the particle core or conjugated externally conferring nanoparticle biocompatibility, target specificity or environmental sensitivity. This study details the development and characterisation of stable, bright, dye-doped silica nanoparticles which are surface functionalised with PAMAM dendrimers to enable efficient conjugation to platelet activation-specific antibodies. We present the physical and optical properties and demonstrate colloidal stability. We also provide the first evidence of how NPs can be employed to specifically label human platelets immobilised on a lab-on-a-chip platform. Using a single step protocol, we demonstrate highly specific platelet labelling with the distribution of antibody-conjugated NPs matching that expected for the platelet GPIIb/IIIa receptor. The work highlights the potential of functionalized fluorescent NPs as diagnostic tools for cardiovascular disease.

From the Clinical Editor: This study details the development and characterization of PAMAM dendrimer functionalized, stable, and bright dye-doped silica nanoparticles that enable efficient conjugation to platelet activation-specific antibodies. These fluorescent NPs may specifically label human platelets that can be used as diagnostic tools for cardiovascular disease.

Key words: Nanoparticle; Fluorescent; PAMAM; Platelet

Platelets play an essential role in normal haemostasis and thrombosis. Damage to endothelial tissue exposes collagen at the site of injury where platelets adhere and a thrombus forms.[1] While haemostasis is a normal response, platelet mediated thrombosis is associated with significant morbidity and mortality in disease states such as myocardial infarction and stroke.[2,3] The rapid assessment of platelet physiology is important to monitor the effect of anti-platelet agents and to predict the risk of adverse cardiovascular events in patients at risk. There are numerous methods currently available to detect and monitor platelet activation including turbidity and impedance aggregometry, flow cytometry and commercially available techniques.[4] Integrating platelet assays into reliable point of care (POC) diagnostics is highly commercially attractive as demonstrated by the VerifyNow® system range.

Bio-diagnostic 'lab-on-a-chip' devices often utilise an analyte sensitive platform, on which both immobilisation and detection occurs which is coupled to a means of identification, commonly through the use of a contrast agent such as a fluorescent reporter. Increasingly, fluorescent nanoparticles (NPs) are used as an alternative to single dye labels due to their high brightness, ease of synthesis and potential for functionalization,[5] with the potential to lower the limits of detection (LOD).[6] NPs can be synthesised from a variety of precursors including silica, noble metals, polymers, self-assembled polymers, magnetic metals,[7-9] with highly customisable features such as size, shape, porosity, surface functional groups and electrical potential.[10] They are attractive as analytical tools as they present a 3 dimensional architecture for incorporation (both internally and externally) of drugs, enzymes, targeting molecules, and contrast agents such as fluorescent molecules. This broadens the strategies for analyte sensing to include the encapsulation of previously incompatible

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dyes such as inorganic, hydrophobic or unstable contrast agents, all of which can be concentrated and protected inside the core of a NP whose surface can be customised to transport the particles to the specific site of interest. It is this multiplicity of function which enables the development of a new breed of tunable, target specific, high brightness, robust NP based sensing platforms which is the main focus of the work presented here.

Some key limitations when employing silica NPs within bio-diagnostic devices is their size and propensity to aggregate in physiological buffers.[9,11] Mono-disperse silica NP suspensions can be maintained through the high electrokinetic surface charges. However, these forces become disrupted in high salt solutions such those in physiological buffers, serum or complex organic solutions such as whole blood. The result is particle aggregation,[12] which can be overcome by modifications to the particles surface introducing either non-reactive groups[12] or large molecules to maintain high zeta potentials, for example polyelectrolyte chains[13] or multivalent dendrimers.[14] In this work, effective colloidal stability has been achieved through the use of poly(amido amine) (PAMAM) dendrimers which greatly improve particle monodispersity (particle stability) whilst providing active binding groups for conjugation to proteins.[15]

The main objective for this work is the development of stable, bright, dye-doped silica NPs for future use in a fluorescence-based platelet assay for fast and effective POC diagnosis of platelet activation state. The NPs are doped with the near infrared dye 4,5-Benzo-5′-(iodoacetaminomethyl)-1′,3,3,3′,3′-pentamethyl-1-(4-sulfobutyl)indodicarbocyanine (commonly known as NIR664) and are surface functionalized with PAMAM dendrimers thus enabling conjugation to platelet activation-specific antibodies. The key features of this paper are (i) the comprehensive characterisation of the NPs using a range of complementary techniques in terms of their optical and colloidal properties and (ii) demonstration of target specificity and platelet labelling.

Methods

NP synthesis

Particles were synthesised by the microemulsion technique[16] in 15 ml cyclohexane by addition of 3.78 g Triton X-100 and 1.6 ml hexanol, 2 ml of conjugated dye and 0.96 ml Milli-Q water and stirring for 5 mins. Following formation of the microemulsion, 200 μl TEOS was added and stirred for 30mins after which 120 μl NH_4OH was added to the solution and stirred for 24 h. The silica shell was added by a further addition of 150 μl of TEOS. Particles were washed four times in ethyl alcohol at 9000 rpm for 25 mins before suspension in ethyl alcohol at 2 mg/ml. All aspects of particle synthesis, functionalization and characterisation are supplied in the supplementary information.

Protein patterning

Polydimethylsiloxane (PDMS) stamps were fabricated as described by Basabe-Desmonts et al[17] with further detail found in supplementary information.

Human platelet preparation, labelling and imaging

Human platelets were obtained from healthy volunteers by extraction of venous blood into evacuated 4.5 ml tubes containing 105 mM Sodium Citrate (BD Bioscience). Coverslips containing fibrinogen patterned surfaces were placed into individual petri dishes to which was added 1 ml whole blood and rocked at 50 rpm for 30 mins at room temperature. Excess cells were removed by repeated (×3) washes with freshly prepared Platelet Buffer (130 mM NaCl, 6 mM Dextrose, 9 mM NaHCO3, 10 mM Sodium Citrate, 10 mM Tris, 3 mM KCl, 0.81 mM KH2PO4, 0.9 mM MgCl) after which the cells were immediately fixed by addition of 3.7% paraformaldehyde for 30 min, washed in 3× PBS and used immediately for cell labelling or imaging. All aspects of platelet purification, immobilisation, labelling and imaging can be found in the supplementary information.

Analytical techniques

Detailed information on all analytical techniques used can be found in the supplementary information.

Results

Overview of NP synthesis and functionalization

The two-step core/shell fluorescent nanoparticle (NP) synthesis used in this study has been described previously.[18,19] Briefly, the first stage generates a densely packed silica core containing NIR664 dye molecules shown in stage 1a (Figure 1), to which a second protective layer, without dye, is added as depicted in stage 1b (Figure 1). In Stage 2, surface modification provides functional groups which provide the dual purpose of stabilising the NPs by preventing particle-particle interactions and providing convenient functional groups for conjugation to biomolecules. The final stage involves bioconjugation for cell labelling (Stage 3, Figure 1).

Particle brightness

The NP can accommodate a large number of dye molecules within the core, which contributes to reduced limit-of-detection (LOD) in assays where the NP is used as a fluorescent label. Fluorescence Correlation Spectroscopy (FCS) was used to quantify the relative fluorescence yield (QY) per unit (either molecule or NP) and hence evaluate the particle brightness compared to the single NIR664 dye molecule.

The maximum fluorescence quantum yield (Φ) for a solution of NIR664 is 23%, however following dye encapsulation, the yield decreases significantly due to dye self-quenching or HOMOFRET (Homologous Fluorescence Resonance Energy Transfer) which occurs at higher dye concentrations and is exacerbated by the relatively small Stoke's shift of the dye.[20]

Despite the reduced quantum yield, the NP is still orders of magnitude brighter than a single NIR664 molecule. FCS of freely diffusing NIR664 molecules identified a single species of diameter 0.74±0.08 nm, with a relative molecular brightness of 100.97±5.19 photon counts per second/molecule (cps/mol)