Round Monodisperse Nanodiamonds: Towards Highly Bright, Biologically Inert Probes for Fluorescence Imaging

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ABSTRACT

Fluorescent nanodiamonds (FNDs) are very promising luminescent probes and sensors in biomedical research, possessing unique properties such as extreme photostability and high biocompatibility.

Here we summarize a complex route towards bioapplicable FNDs which includes boosting of ND fluorescence, roundening of each individual particle and also creation of an antifouling polymeric coating on NDs which can be further bioothogonaly modified. Using complex processing of FNDs at various levels we prepared highly bright unique new materials, superior in particle shape and distribution to commonly used FNDs and perfectly suitable for bioapplications and further (bio)modifications.

Keywords: nanodiamond, spherical, biocompatible, fluorescence, imaging

1 INTRODUCTION

Nanodiamond (ND) is a widly used material in contemporary technology including engineering[1], cosmetics[1] or biomedicine[2,3]. ND particles can be turned into fluorescent by irradiation with high energy particles (p^+, He^{2+}, e^-) and subsequent annealing at >600°C[4]. This procedure creates fluorescent point crystal lattice defects – NV centers [5]. The ND fluorescence exhibits excellent characteristics for bioimaging: emission at ~700 nm, large Stokes shift, long coherence times, and, notably, extreme stability towards photobleaching and photoblinking[5,6].

Hence fluorescent nanodiamonds (FNDs) attract a lot of scientific attention as fluorescent imaging probes. The absolute photostability of FND allowed for instance an unprecendent long term tracking of a single ND particle inside living cell[7]. Furthermore, FNDs were visualized in vivo[4,8], where their photostability is crucial for long-term applications. Furthermore, FNDs allow for the construction of unique fluorescent biosensors[9–11], able to monitor functions of ion channels[12] on the cell surface or the activity of single neurons[13].

Although FNDs can be used in such advanced applications, their successful use in living systems is restricted by certain limitations, described in the following paragraph.

High pressure high temperature (HPHT) synthetic pathway yields NDssubsequently modified into highly bright FNDs, essential for above described imaging and sensing applications. HPHT NDs are polydisperse and of irregular shape. These non-uniform FND are impossible to control in living systems since the behavior of any nanoparticle (including ND) – its metabolic pathway, cell internalization and also possible toxic effect, strongly depends on its size and shape[14–19]. Another factor that limits the use of FNDs is their limited colloidal stability in biological fluids[20,21]. Thus FNDs agglomerate and agglomerated particles can accomplish only limited tasks as biological probes or sensors. Finally, biological fluids also contain proteins (globulins, opsonins), which adsorb onto the surface of bare NDsSuch particles are recognized by the immune system and subsequently excreted.

We summarize here our efforts to overcome all above described limitations of NDs. The complex treatment of NDs involves the following steps: Firstly, we use optimized irradiation protocols to get/produce highly bright FNDs[22]. Secondly, by chemical etching[23] or encapsulation[24], we prepare pseudospherical NDs of narrower dispersity. Finally, by surface modifications with polymers, we yield FNDs which are stable in biological fluids, exhibit antifouling properties and can be easily modified with wide variety of molecules using bioorthogonal coupling reactions[24,25].

2 HIGHLY BRIGHT FNDs

Photoluminescence of FNDs comes/arises from nitrogen-vacancy (NV) centres, localized defects in diamond crystal lattice[5,6]. In the NV centre, the carbon

atom is substituted by nitrogen and the adjacent carbon atom is missing. Unique properties of the NV centre (vide supra) predetermine FND to be an extraordinary fluorescent probe and sensor in bioapplications. These applications require highly bright particles with high NV centre concentration. NV centres in ND are created by a two-step procedure. First, ND is irradiated by high energy particles (e^-, p^+, α), which create vacancies in the lattice. During subsequent heating of ND to temperature above 600 °C, these vacancies start to travel through the lattice and when they encounter nitrogen (which naturally occurrs in HPHT ND in ~200 ppm concentration) they form the NV centre. Time and temperature of this annealing step govern effective N-V pairing. We conducted a systematic study, aimed to find the thermal and kinetic optimum for post irradiation annealing of NDs (Figure 1)[22] .Indeed, we revealed the discreet optimum for both time and temperature of annealing. Next we focused on the optimal FND surface termination with respect to fluorescence. Each ND particle is coated on the surface with non-diamond graphitic carbon. By treatment with air or molten potassium nitrate at temperatures over 500 °C, we removed the non-diamond carbon and, thus significantly increased the FND brightness. By combining of both above described procedures – i.e. optimized annealing and subsequent oxidation treatment, we prepared particles approximately one order of magnitude brighter, than those prepared by commonly used procedures.

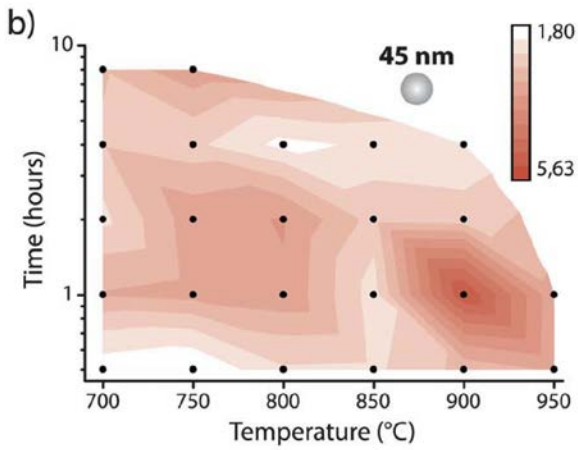

Figure 1. Normalized fluorescence intensity of 45 nm NDs as a function of annealing time and temperature showing the maximum at 900 °C and 1 hour.

3 PSEUDOSPHERICAL NDs

In general, the shape of the nanoparticle is an important parameter, determining its pharmacokinetics as well as pharmacodynamics. For example, distribution, cell uptake, intracellular fate,immune response or even toxicity are all related to the shape of nanoparticles[14–19,26,27]. The spherical shape appears to be the most biocompatible from large

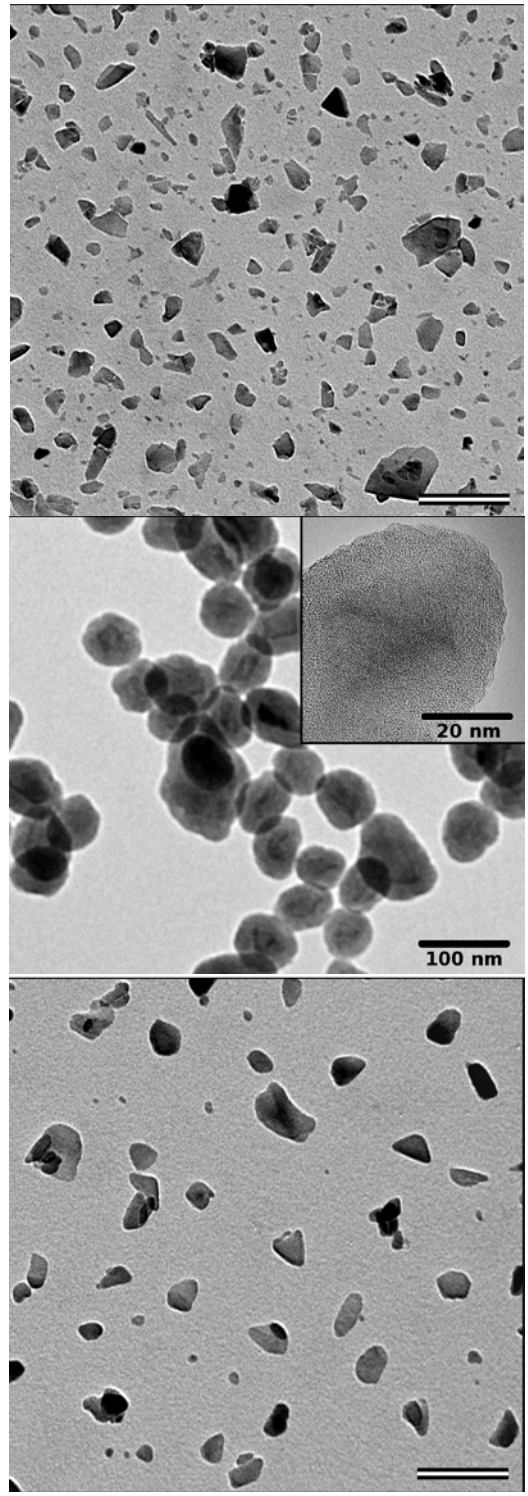

Figure 2. TEM micrographs of commercial NDs (uper), silica encapsulated NDs (middle) and etched, rounded NDs (lower). Scale bars correspond to 100 nm

variety of shapes. High pressure high temperature synthesis of diamonds and their consecutive milling yields polydisperse NDs of irregular shape (Figure 2). In order to achieve more spherical particles, NDs need to be either

encapsulated into spherical shell or the sharp vertexes must be removed from the particles. We use both of these elemental approaches. In the encapsulation, we wrap each FND particle into the shell of silica[24]. The procedure utilizes hydrolysis of silylesters, modification of a method first described by Stöber et al[28]. After coating, the average diameter of FND rises from ~35 nm to ~65 nm and the circularity rises from 0.67 to 0.87 (Figure 2). Furthermore, the dispersity of FNDs narrows upon encapsulation. In the second approach, we removesharp vertexes by etching commercial NDs in molten potassium nitrate[23]. The edges and vertexes of each particle are more reactive than the planes, therefore they are preferentially removed giving rise to round particles (Figure 2). Furthermore, small particles are etched predominantly. As a result, pseudospherical (circularity ~0.79) NDs of mean size ~35 nm and narrow size distribution are obtained.

Both described methods yield pseudospherical particles without sharp vertexes. The behaviour of etched NDs in living systems were studied in vitro and compared with original – angular NDs. The particles behaved differently inside the cell – while the spherical remained in endosomes, the angular were able to escape into cytoplasm[23]. Also the critical cell responses, i.e. apoptosis rate or monocyte activation, were different for angular and etched NDs.

Biological studies clearly show that the shape strongly affects the behaviour of NDs in cells. Hence the ND shape control is essencial/vital for a succesfull design of FND probes and sensors as well as other ND-based biomedical tools.

4 POLYMER COATED FNDS

Bare FNDs without any decoration on their surface have only limited use as imaging probes or sensors because of the following reasons. First, they are not colloidaly stable in biological buffers and agglomerate[20,21]. Second, blood proteins adsorb onto their surface which leads to their recognition the immune system and rapid excretion. Third, it is problematic to attach molecules (e.g. targeting vectors) to their surface. These severe limitations were attempted to overcome by various surface modifications of NDs (for review see ref. [29]). The most promising approach is the modification by a polymeric shell[20,30–32]. We modify FNDs, coated by either thick or ultrathin (>1 nm) functionalized silica shell by biocompatible polymers (Figure 3)[24,25]. The introduction of a silica shell helps/enables a facile effective high-yield modification of FND. Two main approaches were chosen for the introduction of polymers – “grafting to” and “grafting from”[33]. In “grafting to” approach, the polymeric chains are attached to the particles, while in “grafting form” approach, the chains are directly grown from the surface. The polymers contain azide or alkyne moieties, which allow their further modification using Huisgen azide-alkyne cycloaddition (click reaction), an effective bio-orthogonal

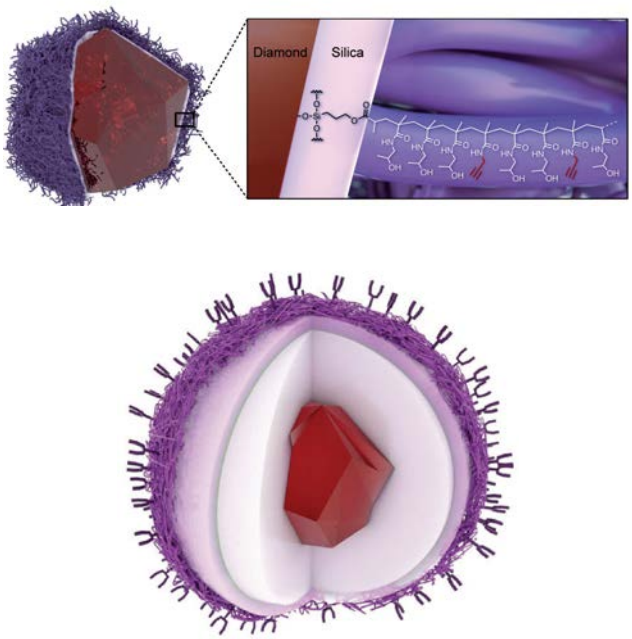

Figure 3. Schematic representation of FND (red) coated with thin (upper) or thick (bottom) silica layer and polymer (violet)

reaction for the attachement of various (bio)molecules to the FND in high yield[34]. Prepared polymer coated NDs are colloidaly stable in buffers (even in 1M NaCl), resistant towards protein adsorption and can be further modified by a wide variety of molecules, which can target them to specific receptors or fulfil other tasks inside the body.

5 SUMMARY

In summary, the simultaneous improvement of FND properties on various levels – boosting their luminescence brightness, normalizing particle shape and size distribution, and coating with biocompatible polymeric shell yields FNDs with superior properties to those currently used. These new particles represent ideal candidates for the most demanding application in fluorescence imaging and sensing.

6 ACKNOWLEDGEMENT

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