PERSPECTIVE

Beyond the Sparkle: The Impact of Nanodiamonds as Biolabeling and Therapeutic Agents

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ABSTRACT A paper by Treussart and co-workers in this issue demonstrates the application of photoluminescent nanodiamonds for intracellular labeling as well as mechanistic cellular uptake studies. Findings from this paper reveal that optimal photoluminescence of nitrogen-vacancy color centers can be attained with photostability and no photoblinking, enabling continuous tracking in the cytoplasm over sustained time scales. In addition to the fluorescent properties of the nanodiamonds, internalization assays reveal a primarily endocytic uptake process. A high degree of nanodiamond (~46 nm in diameter) and endosome colocalization as well as cytoplasmic presence of smaller nanodiamonds was observed. Several attributes of the nanodiamond particles are elucidated in this and other recent studies, ranging from their stability as imaging agents to their potential as intracellular molecular delivery vehicles. These findings give insight into the use of nanodiamonds as an emerging platform for therapeutic and diagnostic ("theranostic") nanomedicine, forging new foundations and criteria for continued nanodiamond engineering toward downstream clinical relevance and impact.

Because of their remarkable properties, nanoparticles comprising carbon, gold, polymers, lipids, and other components have been investigated vigorously for fundamental applications in biology and medicine.[1−6] Nanodiamonds have recently received increasing attention for their potential applications as imaging and drug delivery agents due to several sought-after properties that have been integrated successfully into a unified platform. These include the rapid processing needed to yield uniform and dispersible nanodiamonds that typically include ultrasonication, ball milling, and acid treatment. In addition to impurity removal, acid treatment can also generate surface-bound carboxyl groups for subsequent covalent or electrostatic/physical adsorption of imaging or therapeutic compounds.[7] In addition, as demonstrated by several studies, nanodiamonds can be modified *via* ion irradiation to generate nitrogen vacancy centers with subsequent fluorescence,[8−13] resulting in photostable and dispersible agents for targeted labeling and uptake studies. Furthermore, nanodiamonds can be functionalized straightforwardly with nearly any type of therapeutic, including small molecules,[14−17] proteins,[18,19] and nucleic acids.[20] Nanodiamond surface properties can also promote potent hydration, which could play an important role toward dispersibility of both pristine and functionalized nanodiamonds. While several classes of materials are being developed in parallel as potential solutions for treatment and diagnostics applications, the merger of the nanodiamond platform and its collection of interesting properties, highlighted below, with requisite design considerations may realize a novel, clinically relevant strategy toward nanoenabled medicine.

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Photoluminescence. Biological labeling has been the subject of significant interest for foundational applications in tracking basic cellular processes such as endocytosis, to translational approaches such as cancer diagnostics and imaging. Continued research has sought to develop optimal probes that do not photobleach or photoblink, while remaining biocompatible with minimal additional processing to maintain material scalability. The work by Treussart and co-workers utilized electron bombarded and subsequently annealed nanodiamonds for prolonged time-scale cellular labeling.[21] This work also revealed the presence of smaller, free nanodiamond particles within the cytoplasm without endosomal colocalization, providing a possible size-dependent observation of intracellular release (Figure 1). This observation is particularly relevant toward the rational design of nanodiamond particle sizes and surfaces toward predetermined endosomal escape, which may significantly influence the efficiency of targeted intracellular localization or drug delivery, for example.

See the accompanying Article by Faklaris *et al.* on p 3955.

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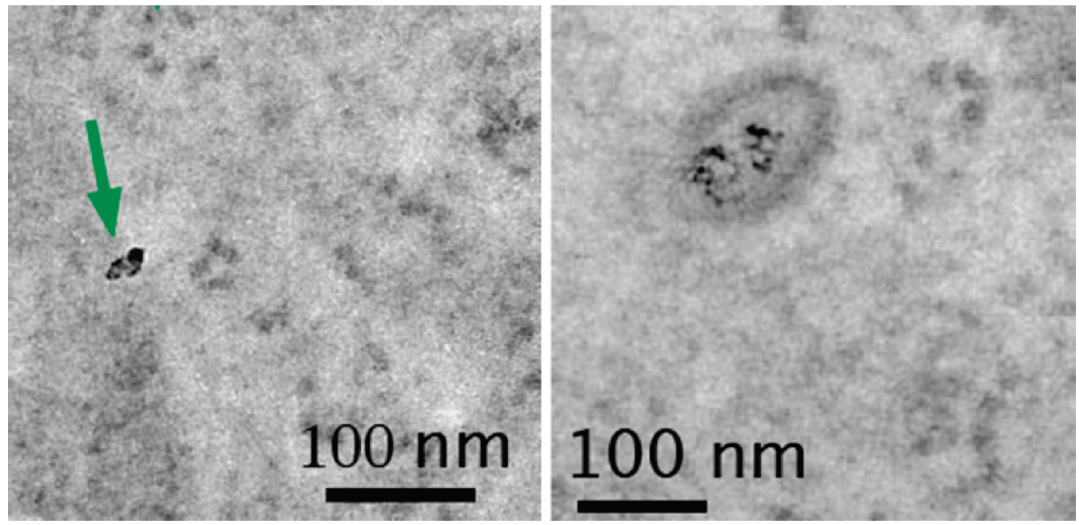

Figure 1. (Left) Treussart *et al*. observed smaller nanodiamonds existing freely in the cytoplasm from potential endosomal escape or passive transmembrane transport. (Right) Intracellular vesicles were also observed to enclose nanodiamonds. Reprinted from ref 21. Copyright 2009 American Chemical Society.

Treussart and co-workers utilized milled diamond microcrystals of approximately 150−200 μm in diameter to generate particles with mean hydrodynamic diameters of approximately 46 nm.[21] Because of the significantly enhanced refractive index value of diamond over cellular environments, it has been observed that the analysis of backscattering signal can result in contrast properties that are conducive toward the sustained imaging of larger diamond particles. As nanodiamond signaling output can be dependent upon particle size, and photoluminescent intensity can be tuned depending upon the number of nitrogen vacancy centers introduced in the nanodiamond, this study utilized fluorescence microscopy to examine the smaller internalized nanodiamonds with tracking capabilities that were sustained in cytoplasmic conditions.

To evaluate the mechanism behind nanodiamond uptake, this study examined both the commonly occurring clathrin-mediated pathway and the caveaolae-mediated pathway. Blocking experiments were utilized to probe each scenario, and showed that the addition of sucrose, which is known to disrupt clathrin-coated vesicle formation, resulted in decreased nanodiamond internalization, while the addition of filipin, an agent used to preclude caveaolae-based internalization, resulted in unaltered nanodiamond uptake behavior. These results strongly suggest that nanodiamonds were taken in using a clathrin-mediated process. Further studies revealing the colocalization of the nanodiamonds with endosomes and lysosomes further confirmed an endocytic mechanism of internalization. Interestingly, smaller nanodiamonds with diameters of 5−10 nm were observed in the cytoplasm, signifying potential endosomal escape, or passive transmembrane movement. Such a finding is important in that the development of design principles for nanodiamond imaging or drug delivery complexes requires the establishment of cellular fate for nanodiamonds of various sizes. As drug delivery efficacy may be enhanced when the therapeutic vehicles are capable of overcoming persistent endosomal encapsulation, the ability to track small nanodiamonds coupled with the finding of small nanodiamond cytoplasmic presence are important discoveries.

With regard to the community of work addressing the high-yield development of photostable nanodiamond probes, Chang *et al*. have previously utilized 40-keV He^+ beam bombardment for the scalable synthesis of nanodiamonds with diameters of approximately 25 nm.[12] This study successfully developed an economical method whereby nearly 2 orders of magnitude increase in yield was achieved using medium-energy ion bombardment that preserved the innate physical properties of the nanodiamonds while simultaneously enabling efficient vacancy generation with lower ion dosing requirements. Subsequent work by Chang *et al*. has functionalized the nanodiamond surfaces with analogues for upregulated cancer markers (*e.g.*, folate) for enhanced cellular targeting capabilities.[22] To expand upon the available wavelengths for imaging, Gogotsi and co-workers developed a nanodiamond−octadecylamine (ODA) conjugate, resulting in a blue fluorescent nanodiamond (Figure 2).[23] Also, Wrachtrup and coworkers recently developed dispersed nanodiamonds with stable photoluminescence, high-contrast optically detected electron spin resonance (ODESR), and radii less than 4 nm.[24]

In examining the rapid emergence of visible wavelength emission nanodiamonds that have been produced using high-throughput methods with consistent photostability properties, a continued push toward the fruition of nano-

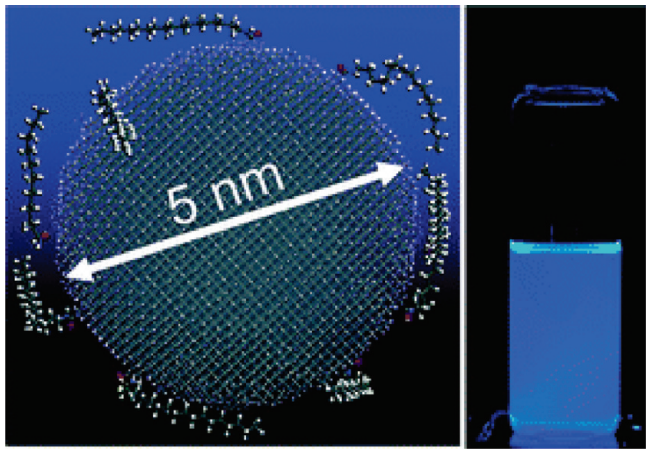

Figure 2. Nanodiamonds can be conjugated to various compounds for labeling and therapeutic applications. Covalent conjugation of octadecylamine (ODA) to nanodiamond surfaces resulted in blue fluorescence, an interesting finding as neither ODA or nanodiamonds alone possessed photoluminescent properties. Reprinted from ref 23. Copyright 2009 American Chemical Society.

diamond-mediated, clinically relevant imaging strategies (*e.g.*, positron emission tomography, computed tomography, and magnetic resonance imaging probes) is an important foundation of transitioning their application from fundamental biology toward medicine.

Surface Properties. Preserved dispersibility remains an important issue when considering the translational efficacy of a potential nanomaterial platform. In fact, nanodiamonds have in fact been previously identified as a lubricant because of their sustained suspension in water and are being explored for applications in the automotive industry. However, solvent conditions in biological environments certainly differ significantly and are indeed dynamic due to the plethora of components and conditions present, from competitive protein binding to changes in pH, among others. Differential scanning calorimetric analysis (DSC) revealed strong electrostatic potentials on the nanodiamond facets, attracting surrounding water molecules and effectively creating a nanoscale shell of water surrounding the diamond surface.[25] In the context of the Miller indices, [111] facets were often characterized as being electrostatically negative while [100] facets displayed positively charged electrostatic fields. Therefore, opposing water molecule alignment resulting from the alternating adjacent electrostatic charges can encourage potent hydration layer adsorption.[25]

In addition to the innate surface hydration properties of the nanodiamonds, Krueger and coworkers developed a beads-assisted sonication (BASD) methodology that dispersed nanodiamond aggregates and subsequently functionalized the resulting primary particles with a model chemical entity. Their strategy activated primary nanodiamond particle surfaces with silane and aryl groups in a homogeneous and consistent fashion as a demonstration of BASD scalability.[26]

Drug Delivery. Beyond biological labeling, nanodiamond surfaces can also be harnessed for therapeutic delivery applications. Previous work has shown that nanodiamonds can be used to mediate sustained release of chemotherapeutics with preserved drug activity. Furthermore, due to the aforementioned high-throughput processing and functionalization steps, drug-nanodiamond binding can be accomplished in an extremely rapid fashion. A recent technological advance has produced a polymeric microfilm device with embedded nanodiamonds for the sustained release of a broad array of therapeutics (Figure 3). In lieu of releasing the nanodiamonds, this device utilized the nanodiamonds as a slow-release matrix, which in addition to enabling long-term elution (*i.e.*, on the time scale of months), also eliminated burst release, an often observed drawback that can result in the elution of the majority of the packaged drug within 24 h, a complication that can result in severe patient side effects. This device could be fabricated at room temperature using chemical vapor deposition, yielding devices of virtually any shape and

Figure 3. Microfilm devices with embedded nanodiamonds are capable of sustained elution of a broad array of therapeutics while eliminating "burst" release. The broad applicability of nanodiamonds as drug delivery platforms may impact several areas of medicine from cancer and inflammation to wound healing. Image courtesy of Robert Lam and Mark Chen.

size needed for localized/implantable therapeutic release.[16]

Beyond the ability to mediate sustained drug release, nanodiamonds have also displayed remarkable enhancements to gene delivery efficacy.[20] Gene delivery is typically characterized by high efficiency and high toxicity, or low efficiency and low toxicity. By functionalizing the nanodiamond surface with polyethylenimine (PEI), a commercially applicable approach toward gene transfection, it was discovered that activating the nanodiamond surface with low molecular weight PEI (PEI800) resulted in a 70× enhancement in transfection compared with PEI800 alone. Furthermore, the inherent biocompatibility of PEI800 was preserved, signifying that both high efficacy and biocompatibility could be manifested in a nanodiamond drug delivery platform.

Biocompatibility. Comprehensive insight into the nature of nanodiamond interactions with the surrounding biology is a critical determining factor of translational significance. At the *in vitro* level, examinations of material biocompatibility can span several levels to include standard laboratory assays for cell viability as an initial view into biological response. Continued studies can utilize quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assays (ELISA) to probe innate cellular gene program activation and protein production/release, respectively, which can provide key information into inflammatory and stress pathway response to nanoparticle presence. *In vivo* studies can examine organ distribution and routes of excretion, among other indicators for nanomaterial safety. Recent studies have explored the localization and removal of nanotubes and organ distribution of larger nanodiamond particles.[27,28] Furthermore, cytokine expression and other quantitative strategies have been employed, supporting the notion of nanodiamond biocompatibility. While cellular response can vary due to the cell lines used, gene programs interrogated, and other parameters such as duration of release, initial nanodiamond studies have provided promising information. Continued work will likely examine long-term organ exposure outcomes and comprehensive examination of the duration needed for complete clearance.

Comprehensive insight into the nature of nanodiamond interactions with the surrounding biology is a critical determining factor of translational significance.

FUTURE ROADMAP

Nanodiamond engineering and chemistry for nitrogen-vacancy introduction and surface processing has realized an exciting platform for biological applications. The study by Treussart and co-workers, as well as other studies described here, have served as a foundation for the requisite attributes for photostable nanodiamond labels, and the dimensional properties that support the cytoplasmic presence of free nanodiamonds. Continued work pertaining to further understanding of surface properties,[29] better control of surface loading/robust grafting and subsequent release of biological agents, nanodiamond agglomeration in solution, and achieving diagnoses capable of output through the skin for medical imaging are examples of key milestones that will inspire new generations of nanodiamond-based platforms with clinical significance. As further suggested by Treussart and co-workers, additional research into controlling cellular fate associated with surface composition, aspect ratios/geometry, size, incubation time, and so forth will elucidate fundamental interaction conditions. In addition, rational design by pairing computation and simulation with experiment will enhance our understanding of drug-nanodiamond interactions and how they can be tuned to optimize drug release and to increase the number of vacancies introduced per diamond, in addition to other critical insights. The corpus of work that is seeding the emergence of nanodiamonds as biologically significant materials is well on its way to catalyzing their significant impact in biomedicine.

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