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Nanodiamonds with Surface Grafted Polymer Chains as Vehicles for Cell Imaging and Cisplatin Delivery: Enhancement of Cell Toxicity by POEGMEMA Coating

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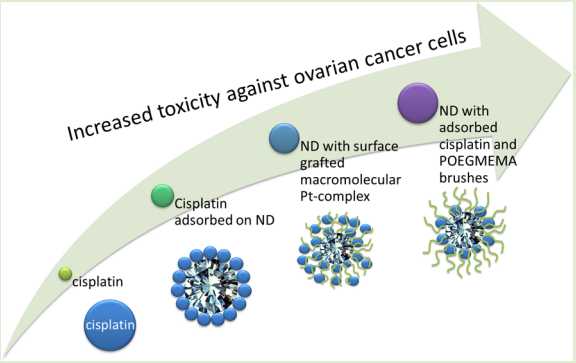
Supporting Information

ABSTRACT: Nanodiamonds (NDs) are highly promising drug carriers due to their biocompatibility, manipulable surface chemistry, and nonbleaching flourescence. In this communication, we compare the cytotoxicity of three ND-cisplatin systems in which cisplatin was incorporated via direct attachment to the ND surface, physical adsorption within a poly(oligo(ethylene glycol) methyl ether methacrylate) POEGMEMA surface coating, or complexation to 1,1-di-*tert*-butyl 3-(2-methacryloyloxy)ethyl)butane-1,1,3-tricarboxylate (MAETC) groups of a POEGMEMA-*st*-PMAETC surface layer. The polymer layers were introduced by grafting from RAFT-functionalized ND particles. All three ND systems displayed lower IC_{50} values than free cisplatin in A2870 and A2870cis ovarian cancer cells. The two polymer-containing systems outperformed their "naked" counterpart, with the POEGMEMA-coated particles the most cytotoxic, displaying an IC_{50} of 1.5 μM, more than an order of magnitude lower than that of cisplatin. The enhanced cytotoxicity is attributed to promotion of cellular uptake by the hydrophilic surface polymer.



The development of new nanomaterials for the diagnosis and treatment of disease has driven enormous progress in nanomedicine, in particular, the delivery of therapeutic agents in a more efficient and targeted manner. Nanodiamond has recently been recognized as a highly promising addition to this class of materials due to its many remarkable properties, including inertness, biocompatibility, ease of functionalization, and nonbleaching fluorescence.[1] In addition, the detonation process used to synthesize nanodiamond particles is inexpensive and produces small primary particles with narrow size distributions. Indeed, the suitability of nanodiamonds as gene and drug delivery agents has been recently demonstrated by several research groups.[2] Enrichment of nitrogen-vacancy defects in the crystal structure of nanodiamonds has been shown to greatly enhance their naturally weak fluorescence to a level that rivals that of quantum dots. Great impetus therefore exists for the development of new surface functionalization approaches that may be applied to bright fluorescent nanodiamonds to generate powerful dual-purpose particles capable of both imaging and therapy.[3,4] Even in the absence of enrichment, the natural fluorescence of nanodiamonds can be used to monitor their location within cells, and remarkably, their unique light scattering properties promote "sparkling" under a light microscope. The combined properties of nanodiamonds, inertness, small size, nonbleaching fluorescent, and high surface functionality,[5] make them the perfect candidate for drug delivery. Their surface structure can be modified and functionalized to conjugate or adsorb various therapeutics and bioactive groups such as DNA, proteins, folates,[6] and other drugs.[4,7] Grafting on the surface can be achieved by noncovalent interaction such as ionic interaction or covalent bonding.[2,8]

Surface functionalization will ultimately lead to changes in solution properties. While many immobilized hydrophilic groups can enhance the solubility of nanoparticles in aqueous solution, the attachment of hydrophobic drugs may lead to a decline in solubility, and aggregation. A hydrophilic polymer layer can enhance stabilization of the nanoparticles in aqueous solution while the layer can also act as a reservoir for drugs. Grafting of polymer chains to tune the solubility and to allow binding of DNA has been reported by Zhang et al.[9] The outcome was highly promising in terms of delivering the payload safely into the cell while enabling imaging. In this communication, the design of a drug carrier for cisplatin based on ND is described. The significant side effects observed during the administration of cisplatin to patients[10] motivated us and other research groups to search for better drug delivery

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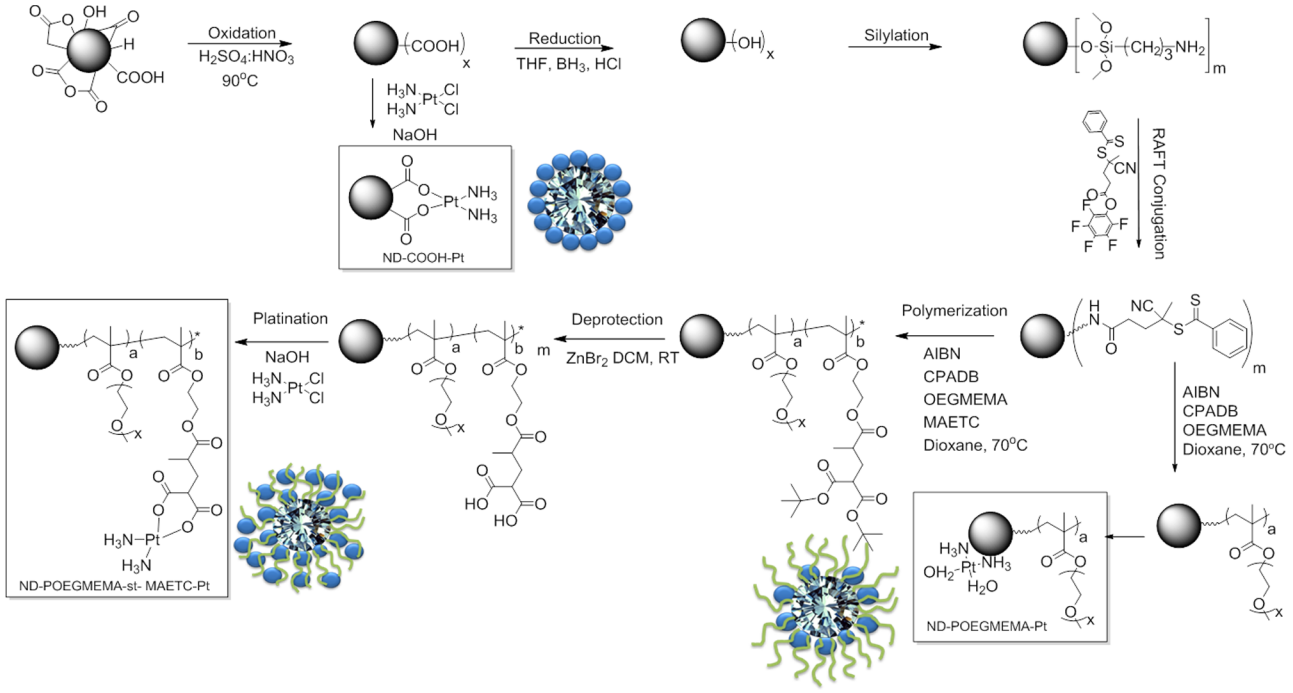
Scheme 1. Synthesis Approach to Three Different ND-Based Drug Carriers for Cisplatin



systems.[11−14] The goal of our platinum delivery systems is to protect cisplatin from deactivation while enhancing its delivery through passive targeting and increasing cellular uptake. For this purpose, the surface of ND was functionalized with polymer chains using the grafting from technique via RAFT polymerization (Scheme 1).[15−18] While ND can possibly be covered with polymers using other techniques since an abundance of functional groups is available on the surface, RAFT polymerization allows the easy control over the brush thickness. This communication focuses on the influence of hydrophilic surface brushes in enhancing the toxicity and effectiveness of ND immobilized cisplatin. POEGMEMA, a water-soluble polymer, was grafted to create a hydrophilic and water-swollen soft layer around the ND. For comparison, a statistical copolymer was grafted that contained POEGMEMA, but also a ligand that can bind additional cisplatin (Scheme 1).

The preparation of the ND surface to allow grafting of polymer chains was performed in four steps. The surface of raw ND aggregates exhibits a variety of functional groups including carboxyl, lactone, ketone, hydroxyl, and alkyl groups, which must be homogenized in order to control subsequent surface reactions and maximize the density of attached molecules. High temperature treatment with oxidizing mineral acids has been demonstrated to be effective in attaining a high coverage of carboxyl groups. Pichot et al. has reported the thermal oxidation of the nanodiamond with nitric acid under pressure[19] and Bradac et al. were able to effectively oxidize the nanodiamond surface via extensive acid washing, using a mixture of sulphuric and nitric acid, and sonication at an elevated temperature of 90 °C.[3] We adopted the latter approach (see Supporting Information).

After attaining dispersed oxidized ND particles, denoted ND-COOH, cisplatin was conjugated directly to carboxylic groups on the surface, generating a polymer-free control against which to compare the polymer-bearing conjugates (see Supporting Information). The procedure employed to complex platinum directly to the surface was similar to that of Guan et al., where cisplatin was immobilized onto ND to test their performance toward human cervical cancer (HeLa) cells (Scheme 1).[20] TGA analysis was used in the current work to determine the amount of immobilized drug loaded on the surface. The platinum residue measured after TGA analysis in air equated to 15 wt %, which, assuming the surface area provided by the ND supplier (40 m^2/g), gives a concentration of 13.6 Pt-complex per nm^2 (Supporting Information, Table S1). The high amount of complexes per area can only be explained either by stacking of complexes. More likely is however that the surface area after the extensive treatment is higher that than the one of the untreated NDs from the supplier.

The carboxylated ND surface required further modification before the RAFT agent could be attached via its R-group[16,21,22] to allow the grafting of polymer chains via RAFT polymerization.[23−25] The reduction of the nanodiamond surface converted the carboxyl groups to hydroxyl groups and improved homogeneity since some tertiary hydroxyl groups originally present on the surface are unchanged by the oxidative process. TEM analysis of the reduced particles confirmed that this procedure is indeed suitable to prevent aggregation (Supporting Information, Figure S1). In addition, the surface modification led to better dispersion and a more transparent aqueous solution (Supporting Information, Figure S1). The measured particle size was determined by DLS to be 95 nm (Figure 1), which is significantly larger than the 10 nm of a single ND highlighting the strong tendency of these ND to aggregate. The aggregation of nanodiamonds has been subject to discussion in the literature, with the consensus reached that the NDs are in fact not aggregated as first suspected, but actually chemically connected. Several procedures have been investigated for achieving full deagglomeration, such as zirconia milling and bead-assisted sonic disintegration,[2] but the vastly improved dispersibility and relatively small aggregate sizes attained in our case did not warrant further processing of the particles. The next step before RAFT attachment was reaction of the surface hydroxyl groups with (3-aminopropyl)-

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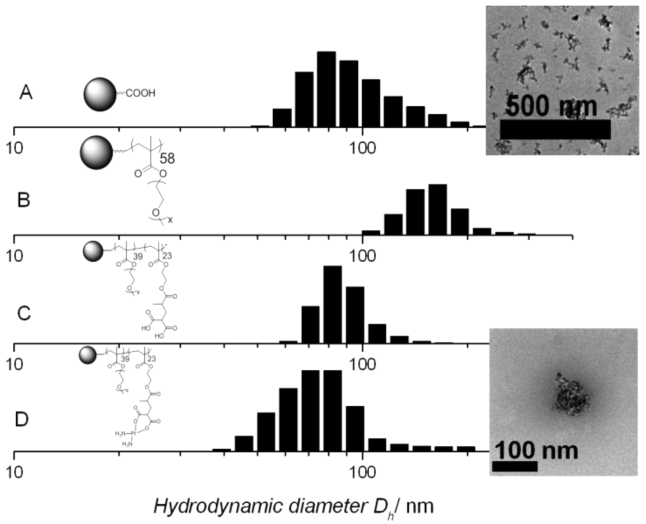

Figure 1. Hydrodynamic diameter in water obtained via DLS of the nanodiamonds at different functionalization steps. TEM images are insets for samples A and D.

trimethoxysilane, which led to amine-functionalized nanodiamonds (ND-NH_2; see Supporting Information, section 2.2.4). Purification was performed by consecutive acetone washing and centrifugation cycles. Finally, reaction of the terminal primary amines with pentafluorophenol-activated RAFT agent (CPADB-PFP) led to ND with RAFT agent immobilized on the surface as depicted in Scheme 1. The amount of RAFT agent on the nanodiamond surface was determined using UV/vis spectroscopy (Figure S2). Although ND itself absorbs in the UV/vis region, a peak at 315 nm corresponding to the RAFT agent is clearly visible, allowing the determination of a RAFT agent density of 0.31 molecules per nm^2 (Supporting Information, section 2.2.5).

The ND-RAFT agents were employed in the polymerization of OEGMEMA. Sacrificial RAFT agent, CPADB, needed to be added to maintain the livingness of the grafting process by preventing the loss of RAFT end groups from the surface. A solution with an [OEGMEMA]/[CPADB]/[AIBN] ratio of 100:1:0.2 was polymerized in the presence of ND-RAFT for 9 h at 70 °C, resulting in a monomer conversion of 58%. A crucial step was the deoxygenation procedure, which was only successful when the solution was immersed in an ultrasonication water bath throughout nitrogen purging. Post-polymerization, the free polymer in solution was analyzed using SEC. The measured molecular weight of 15000 g mol^{-1} (PDI = 1.09) was in good agreement with the theoretical value ($M_{n,theor}$ = 17120 g mol^{-1}), indicating that the process was not affected by the presence of NDs (Supporting Information, Figure S3). The surface-grafted NDs were thoroughly washed to remove any free polymer. High dispersibility in aqueous solution was observed after the grafting of the POEGMEMA layer, with the hydrodynamic diameter of the particles increasing from 95 nm before polymerization to 110 nm afterward (Figure 1). The POEGMEMA was then subsequently cleaved from the ND surface by *tert*-butyl ammonium fluoride and acetic acid and analyzed by SEC. The molecular weight of the recovered POEGMEMA, $M_{n,SEC}$ = 13000 g mol^{-1}, was lower than the molecular weight in solution (Supporting Information, Figure S3). Delayed growth of the grafted polymer compared to the free chains in solution has been frequently observed in literature and can be understood by considering the lower accessibility of the RAFT agent on the surface.[26] Noticeable also is the broadening of the distribution (PDI = 1.18), indicating a slightly uneven growth compared to the polymer in solution, which can also be tentatively attributed to differences in accessibility of the RAFT agent on the uneven surface of the particles (Supporting Information, Figure S3). The amount of polymer on the surface was measured to be 28 wt % using TGA. Considering the molecular weight of $M_{n,SEC}$ = 13000 g mol^{-1} of the surface-grafted POEGMEMA, the calculated grafting density was calculated to be 0.45 polymer chains per nm^2, which is, considering the limits of accuracy in TGA, comparable to the density of the RAFT agent (Supporting Information, Table S2). To test if the NDs with POEGMEMA brushes are capable of adsorbing or physically entrapping cisplatin, the ND were stirred with *cis*-diaquodiamino platinum-(II) and thoroughly washed. TGA analysis revealed a platinum residue of 11 wt %, which equates to 9.9 Pt-complexes per nm^2. Although the surface of the polymer is covered with polymer, the drug is still able to adsorb on the ND surface. Even intensive washing procedures did not result in any noticeable desorption.

Even though the POEGMEMA grafted NDs are capable of adsorbing a reasonable amount of Pt-drug, incorporation of ligands capable of chemically binding cisplatin to the polymer brushes was pursued to enhance the loading capacity. Polymers displaying 1,1-di-*tert*-butyl 3-(2-methacryloyloxy)ethyl)butane-1,1,3-tricarboxylate (MAETC; Scheme 1) groups as macromolecular ligands have been reported in our previous work for the complexation of Pt-drugs to polymers and their successful delivery into tumor cells.[12−14] NDs were grafted to a statistical copolymer of OEGMEMA and MAETC grafted from the surface of the ND using an initial molar ratio of 3 to 2 (Supporting Information, section 2.3.2). OEGMEMA was incorporated to ensure water solubility because PMAETC conjugated with the Pt-drug is insoluble in water. A solution with an [OEGMEMA]/[MAETC]/[CPADB]/[AIBN] ratio of 60:40:1:0.2 was polymerized in the presence of ND-RAFT for 5 h at 70 °C. Analysis of the free polymer allowed determination of the polymer composition (via ^{1}H NMR)[12,13] and confirmed the livingness of the polymerization in solution. The signal at 1.5 ppm correlates to the *tert*-butyl group of MAETC and PMAETC, which was compared to the intensity of the vinyl signal. The conversion of OEGMEMA was obtained by comparing the ethylene oxide signals at 3.5 ppm to the respective vinyl signals. The conversion of OEGMEMA and MAETC was calculated to be 65 and 56%, respectively, corresponding to 39 repeating units for OEGMEMA and 23 for MAETC. SEC analysis confirmed the livingness of the polymerization in solution ($M_{n,theor}$ = 21245 g mol^{-1}, $M_{n,SEC}$ = 17500 g mol^{-1}, PDI = 1.15; Supporting Information, Figure S4). The amount of polymer on the surface of the NDs was determined using TGA. The polymer itself degrades rapidly above 250 °C, while the NDs themselves oxidize at 600 °C (Supporting Information, Figure S5), which is in agreement with the literature.[5] The surface-grafted ND underwent a weight loss of 36% between 300 and 400 °C, which correlates to the surface-grafted polymer (Figure 2). The grafting density was calculated to be 0.40 polymer chains per nm^2, assuming a surface area of 40 m^2/g. Hydrolysis of the *tert*-butyl ester protecting group was carried out using $ZnBr_2$ (Supporting Information, section 2.4.1). The alternative procedures using trifluoroacetic acid resulted in cleavage of the polymer from the surface. The polymer was expected to lose 12.5% of its weight

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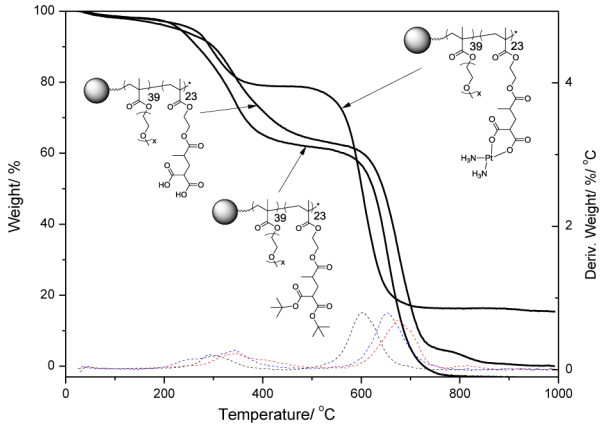

Figure 2. TGA analysis of surface-grafted NDs.

during deprotection (equating to a theoretical polymer mass fraction of 0.3), which corresponds to the approximate mass loss observed using TGA (Figure 2). The subsequent platination was carried out by deprotonation of the carboxylate groups with NaOH, followed by reaction with cis-diaquodiamino platinum(II) (Supporting Information, section 2.4.2). The platination efficiency was also tested using TGA. It is known that the oxidation of cisplatin leads to elemental Pt, which can be used to determine the amount of complex in the polymer. Theoretically, the final product should consist of polymer (25 wt %), ND (65 wt %), and Pt (10 wt %) if the conjugation efficiency is 100%. The actual Pt residue of 13.5 wt % indicates that some cisplatin was adsorbed to the surface of the ND. The possibility of insufficient washing of the ND was eliminated, confirming again that, similar to NDs grafted with POEGMEMA only, the Pt-drugs are adsorbed to the surface of the NDs. The size of ND was determined to be 90 nm via DLS, which matched that observed by TEM (Figure 1 and Figure S6). The hydrodynamic diameters of the NDs before (88 nm) and after (84 nm) cisplatin conjugation are smaller than those of the POEGMEMA-containing particles, which is indicative of the lower water solubility and therefore slightly dehydrated polymer shell. It should be noted here that the NDs with polymer chains attached to the surface seem smaller in size than the "naked" ND with carboxylate functionalities. This reflects the high tendency of NDs to aggregate. Although these NDs are seemingly well dispersed in the solution, they do tend to aggregate again. Shaking or short sonification can reverse the process although this may introduce some errors during DLS analysis.

NDs hold great promise as drug delivery vehicles due to their permanent fluorescence, which does not show any photobleaching over time. In addition, the material is nontoxic as demonstrated in vitro and in vivo.[27] To test the ability of the polymer-grafted ND to deliver platinum drugs, the NDs were incubated with ovarian cancer cells. After a few min, the cells started sparkling due to the presence of internalized NDs (Figure 3). The observations under the light microscope were complemented using fluorescence microscopy. The NDs employed in this study are only weakly fluorescent, but photoluminescent intensity can be increased by enriching the number of nitrogen vacancy centers within the NDs. The bright and fluorescent appearances of the cells confirm the uptake of nanoparticles, most likely via endocytosis.[28]

After confirming successful cellular uptake, the cytotoxicity of the Pt-loaded ND on the ovarian cancer cell line A2780 and the

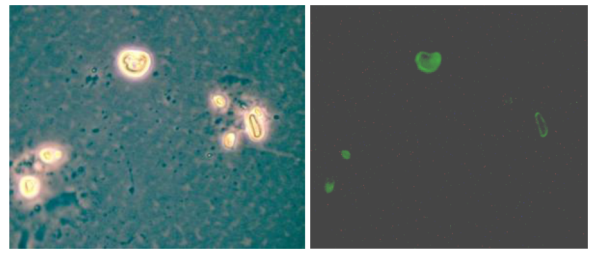

Figure 3. Light microscope (left) and fluorescent microscope (right) images of tumor cells after being incubated with ND-POEGMEMA-*st*-MAETC-Pt at 2 μM for 2 h.

cisplatin-resistant cell line A2870cis was tested. Cisplatin is released from the grafted polymer by a ligand replacement reaction of the carboxylate ligand by chlorides as described in earlier studies.[12,13] A large fraction of the drug is typically released in less than three days.[12−14] Binding of the activated cisplatin to DNA leads ultimately to apoptosis, although some cell lines have developed resistance to cisplatin through a process that is often associated with the increased expression of P-glycoprotein (Pgp).[29] The two Pt-drug-loaded NDs with surface-grafted brushes were compared to ND immobilized cisplatin, which has been investigated by other researchers,[20] and to cisplatin alone. The IC_{50} value was determined from Pt-concentration-dependent cell viability (Table 1 and Figure S7).

Table 1. IC_{50} Values Obtained from the Cell Viability of Two Ovarian Cancer Cell Lines vs the Platinum Concentration

	A2780 (μM)	A2780cis (μM)
cisplatin	16.5	27.6
ND-COOH-Pt	3.8	6.7
ND-POEGMEMA-*st*-MAETC-Pt	2.8	3.7
ND-POEGMEMA-Pt	1.5	1.8

All three NDs increased the cell toxicity compared to free cisplatin. We propose that the cells may have greater difficulty expelling the ND compared to the free drug, which could enhance its cytotoxicity. Free cisplatin molecules can either diffuse or be transported out of the cells by ABC transporter proteins, while ND−cisplatin complexes are more difficult to expel and expose the cell to the drug for a greater period. This effect is particularly pronounced in cisplatin resistant cells where Pgp-transporters are overexpressed.[30] It is also noteworthy that both cell lines responded more favorably to the polymer-coated NDs compared to the polymer-free ND conjugate. It is likely that the PEG shell enhances the cellular uptake, but verification of this hypothesis is the subject of our continuing investigations. It is interesting to note that the POEGMEMA polymer layer is superior to the P(OEGMEMA-*st*-MAETC-Pt) brushes despite possessing a lower concentration of drug. It is likely that the greater water solubility of these particles, confirmed by their larger hydrodynamic diameters (Figure 2), was an important factor in enhancing cellular uptake and thereby increasing cytotoxicity.

In this communication, we showed that grafting of water-soluble polymer chains enhances the therapeutic efficiency of NDs bearing platinum drugs. The IC_{50} value of ND-polymer-Pt was substantially lower than that of both the free drug itself and of ND with the cisplatin drug directly conjugated to the surface. Further studies will investigate the detailed uptake mechanism to understand the origin of this enhancement.

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■ ASSOCIATED CONTENT

Supporting Information

Experimental details and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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