Doxorubicin loaded nanodiamond-silk spheres for fluorescence tracking and controlled drug release

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Abstract: Nanoparticle (NP) based technologies have proved to be considerably beneficial for advances in biomedicine especially in the areas of disease detection, drug delivery and bioimaging. Over the last few decades, NPs have garnered interest for their exemplary impacts on the detection, treatment, and prevention of cancer. The full potential of these technologies are yet to be employed for clinical use. The ongoing research and development in this field demands single multifunctional composite materials that can be employed simultaneously for drug delivery and biomedical imaging. In this manuscript, a unique combination of silk fibroin (SF) and nanodiamonds (NDs) in the form of nanospheres are fabricated and investigated. The spheres were loaded with the anthracyline Doxorubicin (DoX) and the drug release kinetics for these ND-SF-DoX (NDSX) spheres were studied. NDs provided the fluorescence modality for imaging while the degradable SF spheres stabilized and released the drug in a controlled manner. The emission and structural properties of the spheres were characterized during drug release. The degradability of SF and the subsequent release of DoX from the spheres were monitored through fluorescence of NDs inside the spheres. This research demonstrates the enormous potential of the ND-SF nanocomposite platforms for diagnostic and therapeutic purposes, which are both important for pharmaceutical research and clinical settings.

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References and links
1. M. Schafer-Korting, Handbook of Experimental Pharmacology (Springer-Verlag Berlin Heidelberg 2010).
2. X. Zeng, R. Morgenstern, and A. M. Nyström, “Nanoparticle-directed sub-cellular localization of doxorubicin and the sensitization breast cancer cells by circumventing GST-mediated drug resistance,” Biomaterials 35(4), 1227–1239 (2014).
3. E. K. Chow, X. Q. Zhang, M. Chen, R. Lam, E. Robinson, H. Huang, D. Schaffer, E. Osawa, A. Goga, and D. Ho, “Nanodiamond therapeutic delivery agents mediate enhanced chemoresistant tumor treatment,” Sci. Transl. Med. 3(73), 73ra21 (2011).
4. A. Lamprecht, N. Ubrich, H. Yamamoto, U. Schäfer, H. Takeuchi, P. Maincent, Y. Kawashima, and C. M. Lehr, “Biodegradable nanoparticles for targeted drug delivery in treatment of inflammatory bowel disease,” J. Pharmacol. Exp. Ther. 299(2), 775–781 (2001).
5. A. Flórez-Zak, A. Mackiewicz, and H. Damas-Kozlowska, “Functionalized spider silk spheres as drug carriers for targeted cancer therapy,” Biomacromolecules 15(8), 2971–2981 (2014).
6. F. P. Seib, G. T. Jones, J. Rinjak-Kovacina, Y. Lin, and D. L. Kaplan, “pH-dependent anticancer drug release from silk nanoparticles,” Adv. Healthc. Mater. 2(12), 1606–1611 (2013).

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7. H. Maeda, “The link between infection and cancer: tumor vasculature, free radicals, and drug delivery to tumors via the EPR effect,” Cancer Sci. 104(7), 779–789 (2013).
8. J. Liu, M. Yu, C. Zhou, S. Yang, X. Ning, and J. Zheng, “Passive Tumor Targeting of Renal-Clearable Luminescent Gold Nanoparticles: Long Tumor Retention and Fast Normal Tissue Clearance,” J. Am. Chem. Soc. 135(13), 4978–4981 (2013).

9. R. T. Chlebowski, “Adriamycin (Doxorubicin) Cardiotoxicity: A Review,” West. J. Med. 131(5), 364–368 (1979).

10. I. B. Vasconcelos, T. G. Silva, G. C. G. Militao, T. A. Soares, N. M. Rodrigues, M. O. Rodrigues, N. B. Costa, R. O. Freire, and S. A. Junior, “Cytotoxicity and slow release of the anti-cancer drug doxorubicin from ZIF-8,” RSC Adv. 2(25), 9437–9442 (2012).

11. A. M. Rahman, S. W. Yusuf, and M. S. Ewer, “Anthracycline-induced cardiotoxicity and the cardiac-sparing effect of liposomal formulation,” Int. J. Nanomedicine 2(4), 567–583 (2007).

12. K. Kunieda, T. Seki, S. Nakatani, M. Wakabayashi, T. Shiro, K. Inoue, M. Sougawa, R. Kimura, and K. Harada, “Implantation treatment method of slow release anticancer doxorubicin containing hydroxyapatite (DOX-HAP) complex. A basic study of a new treatment for hepatic cancer,” Br. J. Cancer 67(4), 668–673 (1993).

13. C. Harrison, “Anticancer drugs: double boost for doxorubicin therapy,” Nat. Rev. Drug Discov. 13, 178 (2014).

14. P. Horcajada, T. Chalati, C. Serre, B. Gillet, C. Sebrrie, T. Baati, J. F. Eubank, D. Heurtaux, P. Clayette, C. Harrison, “Anticancer drugs: double boost for doxorubicin therapy,” Nat. Rev. Drug Discov. 13, 178 (2014).
DoX after conjugating it to lipids exhibit improved anticancer efficacy against tumour growth be possible due to its slower release [12]. Other studies [12–14] have shown that delivering controlled and progressive release of DoX ensures low cytotoxicity of the drug, which might risk factors enabling its use for long term chemotherapy [9]. Studies have revealed that highly failure [10, 11]. Controlling or reducing the dose of DoX is known to prevent the significant cumulative dose-dependent cardiotoxicity and can lead to drug-induced congestive heart failure [10, 11]. Controlling or reducing the dose of DoX is known to prevent the significant risk factors enabling its use for long term chemotherapy [9]. Studies have revealed that highly controlled and progressive release of DoX ensures low cytotoxicity of the drug, which might be possible due to its slower release [12]. Other studies [12–14] have shown that delivering DoX after conjugating it to lipids exhibit improved anticancer efficacy against tumour growth and reduction in the cardiotoxicity as compared to the drug in free form [15].

Encapsulating the drug in polymer structures [16] ensures controlled release of DoX. Silk fibroin (SF) is a natural biopolymer and has been successfully used in medicine for decades and is highly beneficial in this regard [17, 18]. The surface of SF can be easily functionalized with cell marker proteins [19] making it highly suitable for targeted drug release to specific

1. Introduction
In standard chemotherapy, the key barrier to the treatment of cancer is the resistance of the cells to chemotherapeutic drugs [1, 2]. Anticancer drugs tend to become ineffective because cells reject and pump them out quickly through drug transporting proteins [3]. Furthermore, drug carriers are expected to stay in the blood for an extended period of time and then accumulate at diseased sites [4]. Current studies [1, 3, 5, 6] have identified that nanoparticles (NPs) not only have the ability to be used as targeted drug delivery platforms but they can also overcome drug resistance through the enhanced permeation and retention (EPR) effect [6]. For NP based drug delivery systems [5], the EPR effect facilitates the deposition of nanoparticles at the tumour site due to the tumour’s leaky vasculature, abnormal growth and lack of an effective lymphatic drainage [5, 7]. This results in a higher probability of NPs accumulating at tumour sites. The EPR effect is specific to certain sized components and NPs are known to accumulate at much higher concentrations and for longer duration than drug or dye molecules. These molecules become ineffective because they are rejected due to their small molecular size. Therefore, a larger nano-sized carrier is necessary for the EPR effect to properly delivery a payload [8]. The deposition of NPs followed by their uptake through endocytosis enables the diffusion across the plasma membrane of the cells and effective release of drugs. This increases drug concentration at the target site and is expected to be a more efficient technique than the standard methods of drug delivery.

Doxorubicin (DoX) is a low molecular weight, anthracycline drug which is effective against a wide range of malignant conditions, especially leukemia and lymphoma, as well as cancers of bladder, breast, stomach, lung and ovaries [9, 10]. However, the drug possesses a cumulative dose-dependent cardiotoxicity and can lead to drug-induced congestive heart failure [10, 11]. Controlling or reducing the dose of DoX is known to prevent the significant risk factors enabling its use for long term chemotherapy [9]. Studies have revealed that highly controlled and progressive release of DoX ensures low cytotoxicity of the drug, which might be possible due to its slower release [12]. Other studies [12–14] have shown that delivering DoX after conjugating it to lipids exhibit improved anticancer efficacy against tumour growth and reduction in the cardiotoxicity as compared to the drug in free form [15].

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pathological sites [5, 20]. Additionally, SF is capable of effectively entrapping instead of strongly absorbing the low molecular weight DoX which enables it to diffuse through SF structures [21, 22]. The inherent ability of SF to slowly release DoX is a unique feature to this biomaterial [21]. In addition, SF is biodegradable from weeks to months by modifying its beta sheet content [23, 24]. This tunable degradability allows for controlled drug release from SF structures [23]. Recent studies [6, 21, 22] have revealed that DoX loaded SF structures are able to overcome the DoX resistance mechanisms of breast cancer cells by improving the cellular uptake mechanism. Drug loaded SF structures are able to inhibit cell growth significantly more than the free drug at the DoX equivalent dose [6].

To administer DoX during drug release and to improve tumour detection, the delivery carriers need to be incorporated with biocompatible and robust imaging agents. Intrinsic fluorescence of DoX itself is not utilizable due to its rapid photobleaching characteristics [25]. Generally, SF structures are traced in the biological environment after conjugation with organic fluorophores [5]. However, these fluorophores have the tendency to photobleach quickly, which does not allow monitoring and bioimaging for extended time periods. Most fluorescent dyes are reported to be cytotoxic and are known to induce chemical changes to the biological structures under observation, limiting their use in vitro and in vivo [26]. Moreover, the degradability of SF in biological environment can lead to significant uncertainty in the fluorophore emission. This uncertainty in the emission from the degraded SF makes it harder to determine whether it is the fluorescence from the SF structure where the drug is located or the dye that is being traced [27].

The use of photostable and bright fluorescent NPs is immensely beneficial to incorporate into drug carriers for imaging purposes. Nanodiamonds (NDs) are widely explored fluorescent probes [28] for applications in biology, medicine and imaging. The negatively charged nitrogen vacancy (NV') centre in NDs is found to be brightly fluorescent and extremely photostable at room and body temperature. The wavelengths of excitation and emission for these NV centres, \( \lambda_{ex} = 532 \text{ nm} \) and \( \lambda_{em} = 637-850 \text{ nm} \) respectively, exist in the visible to near infra-red range which, unlike UV doesn’t damage the cells [29] or interferes with the cells’ autofluorescence [30]. In addition, NDs are non-toxic [3], chemically and biologically inert [31] and biocompatible [28]. These characteristics of the NV' centre introduced within NDs make them suitable imaging tools for drug release carriers [32]. One of the most important characteristics of SF is the fact that it combines easily and can be optically activated with various dopants by simply dissolving them with SF such as aqueous suspension of NDs [25]. ND-SF hybrid, structured in the form of spheres or films [33, 34] can be readily detected through optical emission without the use of additional labels or dyes.

Although drug loaded SF carriers have been investigated [6, 21], there have been no studies that reported the dual mixing of drugs and imaging agents in SF nanospheres. Here we report the fabrication and characterization of imaging enabled DoX incorporated SF (NDSX) spheres. We have examined the fluorescence properties of these drug delivery nanospheres through photostable emission of NDs. We have also investigated the potential use of SF to act as a stimulus-responsive drug delivery system [35] in control solvents and a standard protease enzyme model where the enzyme was used to simulate the in vitro and in vivo degradation of SF [36]. The study is highly beneficial in the context of anticancer nanomedicine and targeted drug delivery. These NDSX spheres have the potential to serve as dual functional nanomaterial for drug delivery and subsequent imaging inside the biological structures. The nanosized drug delivery vehicles are expected to offer greater control of the release rates through their biodegradation and enables better understanding of the cell response to the injected drugs.
2. Experimental section

2.1 Silk processing

The protocol for purifying the SF protein is well established and documented in literature [37]. Briefly, the purification of SF initially involved the removal of sericin, by boiling the cocoons in 0.02 M aqueous solution of sodium carbonate (Sigma Aldrich, USA) to remove the undesired sericin molecule. The fibroin bundle was rinsed in deionized water, dried overnight, and then solubilized in 9.3 M aqueous lithium bromide (Sigma Aldrich, USA) at 60 °C for 3 hours. The solution was dialyzed against deionized water (dialysis cassettes Slide-a-Lyzer, Pierce, MWCO 3.5K) which enabled the production of ~6 % w/v silk fibroin solution. The solution was then centrifuged to remove any large aggregates.

2.2 Nanodiamonds

Commercially available detonated NDs (NaBond), with an average diameter of 50 nm were used. The NDs were annealed at 475 °C in order to remove any graphitic layer coating and then dissolved in deionized water to a concentration of 2 mg/mL.

2.3 Sphere fabrication

Aqueous solutions of NDs and DoX were added directly to the SF solution. The final working solution was 0.5 mg/mL of the above mentioned NDs, 10 mg/ml SF solution and 0.5mg/mL of DoX. The working solution was injected through the inner needle of a co-flow device [38] at a flow rate of 40 μL/h and spheres were collected in polyvinyl alcohol (PVA). PVA was the continuous phase, flowing through the outer needle at 4 mL/h, separating the working solution from the bulk forming consistent diameter spheres. After 2 hours the 80 μL volume of NDSX spheres suspended in 8 mL PVA was dried overnight and then centrifuged twice in water to remove PVA. The spheres were then suspended and incubated in 1 mL of the desired solvent. This process was repeated 5 (x3) times in order to incubate and suspend the spheres in control: water and phosphate buffer saline (PBS) and three increasing concentrations 0.1 mg/mL, 1 mg/mL and 10 mg/mL of Protease XIV enzyme (P5147, Sigma). The protease was used to model the in-vivo degradation of SF and its effect on the drug release kinetics.

2.4 SEM

The spheres were incubated at 37 °C and after a period of 24 hours the released drug was extracted and the spheres were re-suspended in fresh PBS solution. This procedure was repeated for 7 days in order to study the surface characteristics of the spheres during drug release. Each batch of spheres was examined under a Zeiss EVO MA 10 (Carl Zeiss SMT, UK) Scanning Electron Microscope (SEM) at 3keV. For higher magnification images, a Supra55VP FESEM (Zeiss) using the SE2 detector at 4kV was used. Each sample was sputter coated with palladium/gold before imaging.

2.5 Particle size measurement

The diameters of NDSX spheres were calculated before and during drug release using MATLAB imaging and measurement tools. The SEM images for each sample were imported to MATLAB using the “Imtool” image processing command. From the image toolkit bar, the “Measure distance” option was selected and the length of the scale bar (along x-axis) in pixels was determined for the SEM scan at a given magnification. The factor was used to convert the diameters of individual particles to nm. A number of particles (25-40) were selected for each sample and the size distribution was determined.

2.6 Confocal fluorescence microscopy

Spheres suspended in the respective solutions were cast directly on glass coverslips and analysed with the commercial confocal laser scanning microscope (SP2, Leica Microsystems)
in transmission mode. Each coverslip was inverted and placed on the galvano-stage of the microscope. A small oil drop was placed on the back of the coverslip and the 40 ×, 1.25NA oil immersion objective was immersed in that drop. Argon laser was used and 514 nm laser line was selected using acousto-optical beam splitter to excite the NDs. The photomultiplier tube (PMT) detector with an 8-bit output was used to detect the fluorescence within the NDs detection band of 630-800 nm. The gain of the PMT was selected to be 550 V. The laser scanner was set at 20-Hz line scans to ensure slower and brighter images at a scan resolution of 512 × 512 pixels. A line average of 2 and scan average of 2 were used to prevent the unwanted background noise. To avoid any additional noise, background counts for each sample were also recorded and compared to the counts obtained for individual particles.

2.7 Drug release

DoX release experiment was performed for NDSX spheres in control solvents (deionized water, PBS) and Protease XIV (P5147, Sigma) enzyme. The spheres were suspended in five (× 3 each) different solutions (i) water, (ii) PBS, (iii) protease 0.1 mg/mL (pro 0.1), (iv) protease 1 mg/mL (pro 1) and (v) protease 10 mg/mL (pro 10) concentrations. The released drug was monitored with fluorescence spectrometry. The spheres were incubated and rotated uniformly at 37°C (Incubator, Enviro-Genie) with continuous mixing and agitation. The release of DoX into the solvent was measured every 24 hours and the process was repeated for 14 days. After 24 hours, the fluorescence of released DoX into the suspension was measured by centrifuging the spheres and extracting the DoX dissolved supernatant. The residual spheres were re-suspended in fresh solvents and incubated. To get good statistics, three different supernatants for each of the five solvent were pipetted into a 96-well fluorescence microplate. The well plate was inserted to the microplate reader (SpectraMax M2, Molecular device) and measurements were made using the fluorescence intensity detection modality. The samples were illuminated with the absorption peak of DoX at 480 nm. Fluorescence was detected at the peak emission of DoX at 570 nm. A calibration curve was plotted to relate the fluorescence intensities to the known amounts of DoX through curve fitting parameters [39, 40]. The parameters from the calibration curve were used to find the unknown concentrations of released DoX in the supernatant corresponding to the fluorescence intensity in the supernatant after every 24 hours.

3. Results and discussion

The manuscript reports the fabrication, Section 3.1, structural, Section 3.2, and optical characterization, Section 3.3, of NDSX spheres. The drug release from the spheres was monitored with fluorescence variation in Section 3.4. The structure and size of sphere during drug release was investigated by scanning electron microscopy (SEM) and the fluorescence was tracked via confocal fluorescence microscopy. DoX release resulting from the degradation of SF spheres was monitored in control and in a proteolytic model with increasing concentrations as presented in Section 3.5.

3.1. Synthesis of spheres

The fabrication of NDSX spheres was performed through the already developed co-flow technique [38]. The technique involved an all aqueous processing at room temperature, unlike various other techniques of sphere fabrication which expose the drug or SF to heat, pH extremes or freezing and drying [24]. These conditions not only influence the surface of spheres but may also affect the drug release kinetics [23, 24, 38]. For the sphere fabrication, calculated amounts of SF, DoX and NDs in aqueous solutions were mixed together and flowed through the inner discrete phase channel at a flow rate of 40 µL/h of a co-flow capillary device. The spheres produced were dispersed in PVA flowing through the outer continuous phase channel at a flow rate of 4 mL/h. The sphere diameter is controllable and depends on the parameters set for the co-flow device and the concentration of SF used for
sphere synthesis. The spheres were washed and centrifuged twice to remove any residual PVA and then suspended in three different solutions: the control solvents (i) water, (ii) PBS and three different increasing concentrations of protease enzyme as the physiological solution: (iii) pro 0.1, (iv) pro 1 and (v) pro 10. Water was used as a control solvent and PBS as the solvent for the in-vitro drug release model to mimic blood serum and extracellular fluid. Different concentrations of protease, similar to the concentrations found in the cytoplasm of cells mimic the intracellular fluid, were examined as a more effective model to simulate the in-vivo degradation of SF.

3.2. Surface analysis

The surface and morphology analysis of the spheres was performed using SEM. Spheres suspended in PBS solvent were selected as representative sample for surface study. The degradation of spheres followed by the subsequent release of drug was monitored at different points for 7 days via SEM. This was performed in order to determine any changes in the surface morphology and the size of the spheres during degradation. Initially, the SEM images of the NDXS spheres before any drug release on day 0 were recorded as shown in Fig. 1.

Fig. 1. (a), (b) SEM images for NDSX spheres at day 0 before incubation at 37 °C for drug release. The scans were taken at a magnification of 20 k.

The spheres were found to be spherical and porous as can be seen from the figure. Using MATLAB, the size of the spheres was estimated and found to exist in a broad range from 300 ± 40 nm for the smallest to 1200 ± 50 nm for the largest diameter sphere. The mean of the distribution calculated for 29 individual NDSX spheres on day 0 was estimated to be 660 nm. To investigate the degradability of SF and effect of drug release, the incubated spheres in PBS were extracted and deposited on silicon (Si) substrates on day 1, 3 and 7 during drug release and surface characterized with SEM. Figures 2(a)-2(f) show the SEM images of NDSX spheres in PBS on days 1, 3 and 7 during drug release. In Fig. 2(a)-2(b), it may have occurred that there was some residual PVA left in the spheres (even after centrifuging in deionized water) as evident in the SEM images for the day 1. But on subsequent days of drug release, the PVA was washed away completely during solvent change and hence the spheres appear individually and free of any PVA residual in Fig. 2(c)-2(f). The sphere sizes were estimated for each day using MATLAB. The average sphere diameter was found to decrease with time as evident from the figure. Figures 2(a) and 2(b) show the SEM images for spheres after first day of drug release at two different magnifications of 10 k and 20 k. The size distribution for 29 individual particles was measured with MATLAB and the diameters were found to exist in a range of 290 ± 15 nm for the smallest and 800 ± 45 nm for the largest sphere with a mean value of 520 nm.
This means that after one day of incubation and drug release, the average diameter of the spheres was reduced by 140 nm. Figure 2(c) and 2(d) show the subsequent scans of the spheres on day 3 of drug release at 10 k and 20 k magnifications. For the distribution of 42 NDSX spheres on day 3, the diameters were measured to vary from 95 ± 3 nm for the smallest sphere to 450 ± 10 nm for the largest ones, with a mean value of 250 nm, resulting in an average decrease of 270 nm as compared to day 1. At day 7 of the drug release the SEM scans were taken again and are shown in Fig. 2(e) and 2(f) at magnifications of 75 k and 100 k. On day 7, the diameters were measured for 25 residual particles and found to exist in a narrow range of 25 ± 1 nm for the smallest to 60 ± 2 nm for the largest particle with a mean value of 41 nm. The distribution with a standard error of ± 10 nm falls in the range which matches
with the average ND size of 50 nm (as mentioned in Section 2.2). This means that in seven days period, most of the SF was degraded leaving the NDs in the suspension. The change in diameter of the spheres identified that the individual layers of SF fragmented from the exposed surface area. The fibroin diameter continued to decrease slowly with time, leading eventually to complete degradation of SF.

3.3 Fluorescence tracking of spheres

Emission properties of NDs in NDSX spheres were compared with bare NDs and DoX-SF spheres before drug release on day 0. Fluorescence imaging was also performed at three different points during drug release. Spheres suspended in PBS were cast directly on glass coverslips at day 0, 1, 3 and 7 during DoX release. The samples were dried and then analysed with the confocal laser scanning microscope. On day 0, the NDSX spheres showed fluorescence as can be seen in the 8 × magnified confocal scan of Fig. 3(a). Figure 3(b) shows the NDs alone imaged at 8 × magnification. The marked regions contain individual particles that were further characterized for emission intensity analysis. Comparing the Figs. 3(a) and 3(b) clearly shows that the NDs fluoresced brighter when encapsulated in SF spheres as compared to without encapsulation. The sphere size in the fluorescence images was also larger compared to NDs, as predicted by SEM images in Section 3.2. Finally, DoX-SF spheres were illuminated with the same excitation conditions and a much fainter fluorescence was observed as shown in the 8 × magnified fluorescence image of Fig. 3(c).

![Fluorescence images of NDSX spheres at 8 × magnification before drug release on day 0.](image)

The emission counts for the above three samples were recorded for individually selected particles of Fig. 3(a)-3(c) and plotted against time in Fig. 4(a)-4(c). Fluorescence in a range of 75.5 ± 1.5 to 190.0 ± 2.7 A.U. (arbitrary units) was observed for NDSX spheres as can be seen in Fig. 4(a). The spheres that had a size approximately equal or less than a micron on the fluorescence map were selected for the emission counts recorded. The emission intensities of NDs on their own were also recorded and the counts existed between 39.6 ± 0.3 to 45.5 ± 0.6 A.U. range as plotted in Fig. 4(b). DoX-SF spheres (without NDs) were illuminated with the same excitation conditions and a much fainter fluorescence in the range of 19.6 ± 0.2 to 23.7 ± 0.3 A.U. was observed as shown in Fig. 4(c). The concentration of DoX used in DoX-SF spheres was the same as the amount used for NDSX spheres. In addition, as compared to the highly photostable emission of NDs, shown in Fig. 4(b), the fluorescence from DoX alone was found to be strongly photo-bleaching, decaying to the background value within a minute, as illustrated in Fig. 4(d). The low level of fluorescence from DoX-SF spheres and DoX alone confirmed that the bright emission from NDSX spheres resulted from the encapsulation of NDs within the SF spheres. Enhancement in emission of NDs was attributed to the change of the local optical environment seen by the emitted photons. The refractive index of the
surrounding interface changed from $n_A = 1.0$ (air) to $n_{SF} = 1.54$ (SF) for NDs alone and SF encapsulated ND spheres respectively [41]. Hence the increase in emission came from reducing the refractive index difference between the diamond NP, $n_D = 2.4$, and the surrounding environment. The enhancement of fluorescence intensity has been reported previously for NDs coated with SF film and spheres [33, 34]. The brighter and photostable emission from varying diameter NDSX spheres on silica substrate was compared to numerical calculations as shown in Table 1. A 3-dimensional Finite-Difference Time-Domain method was applied using commercial software RSoft to measure the ratio of emission from SF sphere encapsulated NDs with respect to emission of bare NDs on the silica substrate. As evident from Table 1, the emission increased with increase in the size of the encapsulating SF sphere both for parallel and orthogonal polarizations of the ND. The two extreme polarizations were considered with respect to the substrate. The calculations provided the highest emission enhancement for sphere size ranging from 100 to 600 nm, when the ND inside the sphere was positioned closest to the surface of the substrate. Hence the enhancement increased with the proximity of the ND with respect to the substrate.

Table 1. Emission enhancement for NDs inside SF spheres with respect to bare NDs.

| Position of ND | 100 nm sphere size | 300 nm sphere size | 600 nm sphere size |
|----------------|---------------------|---------------------|---------------------|
| Orthogonal     | Parallel            | Orthogonal          | Parallel            | Orthogonal          | Parallel            |
| Closest to substrate | 1.67 | 1.55 | 1.97 | 2.98 | 1.98 | 3.41 |
| Centre of sphere | 1.30 | 1.42 | 1.37 | 2.75 | 2.01 | 3.75 |
| Top of sphere | 0.95 | 1.24 | 0.76 | 2.56 | 0.83 | 3.16 |

Fig. 4. Emission intensity in arbitrary units versus time plots for (a) NDSX spheres, (b) NDs alone and (c) DoX-SF spheres. The red data represents the background in the images. (d) Emission from DoX only dropcast on coverslip, showing rapidly photobleaching intensity.

The experimentally observed enhancement existed in a range of ~2-4 times for different SF coated ND spheres that showed varying diameters (from 300 nm to <1 µm), ND
polarization and distance from the substrate. It should also be noted that a single SF sphere might contain more than one ND, which accounted for slightly larger experimental enhancement values. The number of NDs inside a SF sphere cannot be quantized for the co-flow method utilized for sphere fabrication. In the current method, the suitable concentrations of NDs, DoX and SF were mixed uniformly together and then injected to the system for sphere fabrication. However from the initial concentrations used, the ratio of NDs to SF by weight was 1:20. To be consistent with the fluorescence measurements, we were careful to select the smallest spheres only that increases the probability of containing single NDs.

3.4 Fluorescence variation with drug release

SF-based delivery carriers often show drug release characteristics that are governed mostly by solubilisation or degradation of the SF [6]. For the present case, the degradation of SF reduced the sphere size as shown in Section 3.2, affecting the emission properties of NDs on the encapsulated spheres which was investigated during the drug release. For this purpose, the fluorescence variation from the spheres suspended in PBS and pro 0.1 was monitored. A small volume (5 µL) of the spheres suspended in PBS was drop cast on glass cover slip and imaged at 4 × magnification at day 1, 3 and 7 during the drug release, as shown in Fig. 5(a), 5(b) and 5(c) respectively.

![Fluorescence images of NDSX spheres (suspended in PBS) drop cast on glass coverslip at 4 × magnification during drug release on (a) day 1, (b) day 3 and (c) day 7. The marked regions were used for emission intensity analysis. The brightness scales from a minimum of 0 to a maximum of 256.](image)

The emission intensity versus time for these spheres on day 1, 3 and 7 during drug release are plotted in Fig. 6(a)-6(c) respectively. The figure reveals that the fluorescence intensity of individual spheres decreased significantly as SF degraded. The reduction in sphere size and decreased emission were also indicated by SEM images of Fig. 2 and numerical calculations of Table 1 respectively. The intensity varied from an average value of 70.0 ± 2.9 A.U on day 1 to 50.1 ±2.3 A.U and finally to 29.4 ± 0.9 A.U. on day 3 and then day 7 respectively. The spheres suspended in pro 0.1 were also checked for the fluorescence variation during drug release period. A similar behaviour of fluorescence reduction was recorded for the spheres. Figure 7(a)-7(c) shows 4 × magnified fluorescence maps of spheres suspended in pro 0.1 drop cast on glass coverslips on day 1, 3 and 7 days during drug release. The intensity record of the selected spheres from each of these three scans is shown in Fig. 8(a)-8(c). The intensity reduced significantly from an average value of 86.0 ± 1.7 A.U. on day 1 to 53.0 ± 1.6 A.U. on day 3. However after the 3rd day, the intensity range appeared to be similar, averaging to a value of 56.0 ± 2.2 A.U. on day 7. This indicated that SF spheres proteolytically degraded faster followed with reduction in fluorescence in the first 3 days, as discussed in Section 3.5. This effect can also be noted in Fig. 9. Rapid degradation of silk spheres might have resulted in similar fluorescence intensity range on day 3 and 7, due to low encapsulation of NDs with SD. Hence the reduction in size of the spheres, as presented from SEM measurements, caused
a subsequent reduction to the fluorescence of NDs due to the degradation of SF spheres encapsulating the NDs.

Fig. 6. Emission intensity (in arbitrary units) plots for NDSX spheres in PBS during drug release on (a) day 1, (b) day 3 and (c) day 7. The intensity of the dark region in the background is shown with red connected dots.

Fig. 7. Fluorescence images of NDSX spheres (suspended in pro 0.1) drop cast on glass coverslips at 4 × magnification during drug release on (a) day 1, (b) day 3 and (c) day 7. The marked regions were used for emission intensity analysis. The brightness scales from a minimum of 0 to a maximum of 256.
3.5 Drug release kinetics

Finally, SF spheres were incubated in three increasing concentrations of Protease XIV at 37 °C to simulate an in-vivo model system of proteolytic degradation and study the consequent drug release. The spheres were incubated and suspended in control (water and PBS) and proteolytic solvents to monitor the drug release concentration followed by the degradation of SF spheres. The DoX release experiment was performed for two control solvents and three concentrations of protease samples with fluorescence spectrometry. The spheres in each solvent were incubated and rotated uniformly at 37 °C with continuous mixing and agitation. Three replicates for each sample were prepared to get a spread of data for each of the five solvents every day. The average amount of drug released each day and the standard deviation from this average value was calculated from the spread of data for each solvent. The cumulative drug release percentage of DoX into the solvent was measured every 24 hours for 14 days. The experiment revealed rapid release during the first 7 days and then slower release for the remaining 7 days. The percentage of the released drug was measured and accumulated drug amounts on each successive day are plotted in the Fig. 9 for each of the five solvents. For cumulative drug release, the errors were calculated using standard expression for error propagation [42]. These standard deviations were then plotted as error bars in MATLAB. The total amount of DoX released was compared in Fig. 9 for the proteolytic solvents and the control samples. Incubation of NDSX spheres in protease enzymes led to higher drug release indicative of increased overall degradation of SF over time. The total cumulative drug release from was 27 % from water, 12 % from PBS, 30 % from pro 0.1, 65 % from pro 1 and ~100 % from pro 10 in 14 days.
All measurements were background subtracted from the respective solutions. On day 13 and 14, the fluorescence from pro 10 matched with the background fluorescence of pro 10 alone, which indicated that all the DoX was released into the solvent and the spheres were degraded completely due to the high concentration of protease enzyme. This was used to estimate the loading of DoX in NDSX spheres, found to be ~50% of the input value. From the initial concentration of DoX used in the starting mixture, the expected amount in spheres was 40 µg/mL but the value reduced to ~20 µg/mL in the final NDSX spheres suspension. Furthermore, the plot of Fig. 9(a) suggested that spheres incubated in PBS degraded at the slowest rate. The DoX release was faster in water compared to PBS due to their differences in ion concentration leading to variations in osmotic pressure. Deionized water has a lower ion concentration possessing a lower osmotic pressure leading to a faster DoX release rate compared to PBS. Moreover, PBS simulates a more accurate fluid medium for DoX release providing a longer release if tested *in-vivo*.
that the first two cuvettes (from left to right) with water and PBS contained DoX (with a characteristic redish pink color) loaded spheres suspended in the solvents even after 7 days. In contrast, the three protease solvents became clearer with every passing day and were almost transparent to pale yellow (protease’s characteristic color) on day 7. The last three cuvettes in Fig. 10(a) to 10(d) showed fewer spheres within the passage of days. The amount of drug released in µg on each individual day of observation is plotted in Fig. 11. Figure 11(a) shows that low amounts of the drug were released in the control solvents while higher drug concentrations were released in protease solvents, as obvious from Fig. 11(b). Drug release was controlled by degrading SF layers from the spheres. However, a noticeable initial burst release of DoX was observed in water (8%), pro 1 (0.5%) and pro 10 (15%) in the first three days, indicative of the enhanced dissolution of the active drugs within the core of the spheres. This effect is normally evident at 37°C [24, 39] and is expected to reduce by using higher SF concentrations for the starting solution [43]. After the first three days, the drug released slowly in a controlled manner within 2 weeks. The cumulative drug release from the spheres was fitted using a semi-empirical power law equation:

\[
\frac{C_t}{C_{tot}} = \frac{C_b}{C_{tot}} + k t^n,
\]

where \(C_t\), \(C_{tot}\) are the amount of drug released at time \(t\) and total concentration of DoX respectively. \(C_b\) is the amount of burst released drug, \(k\) is the release constant and \(n\) is the release exponent.

![Fig. 11. Amount of DoX released per day in water (blue), PBS (green), pro 0.1 (black), pro 1 (red) and pro 10 (magenta). Slow and rapid burst effects can also be noticed for respective solvents.](image)
The values of $k$, $n$ and $C_b$ for the five different solvents are shown in Table 2. The value of $n$ existed in a range of 0.35-0.42, which matched for SF sphere-based release [39]. The error bars in Fig. 11 were calculated using the standard deviation of the data around the mean value.

Table 2. Release kinetics of DoX from NDSX spheres suspended in water, PBS, pro 0.1, pro 1 and pro 10.

| Solution | Release constant $k$ | Release exponent $n$ | Burst amount $C_b$ |
|----------|----------------------|----------------------|-------------------|
| PBS      | 4.8                  | 0.40                 | 0.162             |
| Water    | 10.5                 | 0.42                 | 1.560             |
| Pro 0.1  | 11.7                 | 0.35                 | 0.004             |
| Pro 1    | 21.9                 | 0.41                 | 0.095             |
| Pro 10   | 43.1                 | 0.36                 | 3.018             |

4. Conclusions

The work discussed in the manuscript reported the fabrication of DoX incorporated NDSX spheres and investigated their surface, optical and drug release characteristics. The SEM analysis indicated that the diameter of the spheres was found to decrease with the degradation of SF. NDs inside the NDSX spheres were found to emit brighter fluorescence as compared to bare NDs due to SF encapsulation. The ND-enabled emission from the spheres was highly photostable and the enhancement reduced with the reduction in the sphere size. The drug release kinetics of NDSX spheres monitored in control and protease solvents showed progressive and controlled release over a period of 14 days and the release rates were highly sensitive to the solvent. These NDSX spheres have the potential to serve as dual functional NPs for drug delivery and subsequent imaging inside the biological structures. The biocompatibility of NDs and SF combined with the long term imaging capability of NDs and slow degradation make this hybrid material an excellent drug carrier. While SF spheres enable controlled drug release, NDs would ensure bright and photostable imaging and location tracking of the released drug, for preliminary in-vitro tumor models. The reported hybrid biomaterial spheres are easy to synthesize, applicable to a wide range of drugs and can be easily translated to a number of cancers and tumors. The imaging enabled spheres are significantly useful for the development of localized chemotherapy.

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