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An experimental and theoretical assessment of quantum dot cytotoxicity

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Abstract

Quantum dots (QDs) are a class of semiconductor nanoparticles that possess a unique set of size-tunable optical properties. The potential applications of QDs in biological and medical applications are enormous - some notable examples being in high-resolution cellular imaging, cancer tumour targeting and drug delivery. However, the mechanisms for QD-cell interactions are at best partially understood, and QD cytotoxicity is an ongoing concern. In particular, it remains unclear how QD uptake by cells and subsequent cell fate are influenced by QD parameters such as size, composition, concentration, and exposure time. To help resolve this complex issue in a systematic manner, we have developed here one of the first mathematical models that describes the toxic effects of QDs on cells. The model consists of a system of ordinary differential equations describing (among other things) the transition of healthy cells to an apoptotic or necrotic state induced by QD toxicity. We also experimentally investigated the behaviour of a cell population subsequent to exposure to various types of CdTe QDs. In a population of identical cells exposed to QDs of similar size (2-5 nm), it was found that some of the cells entered apoptosis, others entered necrosis, and others demonstrated no response at all. The toxicity of the various QDs was conveniently quantitatively assessed using the parameters appearing in the mathematical model, and satisfactory agreement between theory and experiment was found.

Introduction

In recent decades, human exposure to nanomaterials has become commonplace. Nanoparticles are now widely used in items such as cosmetics, paints, solar cells, sunscreens and medical devices. Humans are readily exposed to nanoparticles via the airway and contact with skin and mucosal surfaces. As a consequence, nanotoxicology has now become a topic of key importance in modern applied nanoscience. Ingestion of QDs by cells inevitably leads to a number of morphological and biochemical alterations, including cytoplasm granulation, loss of cell functionality, nucleus fragmentation, chromosome damage, and eventually cell death. In order to minimize the damage, appropriate QD composition, doses, exposure time and conditions must be used. To complicate matters further, these parameters may need to be varied for different cell types and animal models.

Once trapped inside vesicles, ingested nanoparticles are transported to different organelles during endocytosis. Invading particles that the cell recognises as being foreign are passed to lysosomes or peroxisomes[1]. Both of these compartments have an acidic and oxidative environment that can break down and destroy ingested foreign species. It has been shown that such conditions result in the elimination of capping surface molecules from QDs leading to the exposure of the bare core of the QDs to the cell[2], and the subsequent release of Cd^{2+} ions into the cytoplasm. Moreover, due to high surface-to-volume ratio, a large number of atoms with unsaturated bonds are available to form free radicals. QD cytotoxicity also depends on particle diameter – small green-emitting QDs (approx. 2 nm) have a greater number of surface atoms and can easily penetrate the nucleus, while larger red-emitting QDs (approx. 5 nm core) are less aggressive and remain within the cytoplasm[3].

The outcome of nanoparticle interaction with a cell depends on many factors including, but not limited to, cell origin, cell line, exposure time, culture conditions, and nanoparticle type. There is a large diversity in the experimental design of biocompatibility studies, and the toxic effects of nanoparticles on numerous different types of cells/organisms have been investigated, from human

cancerous cell lines and immune cells to primary mouse blastocytes and, more recently, primates[4,5]. At a certain concentration/time point, QDs induce cell death, which may follow 3 pathways: apoptosis, necrosis or autophagy. Classically, these mechanisms were thought to be mutually exclusive; however, some studies have shown co-existence of apoptotic and necrotic cells in the same population[6,7].

The mathematical modelling of nanoparticle behaviour in cellular contexts has been largely concerned with developing models to describe particle uptake and subsequent intracellular processing. The majority of these models are at the level of a single cell[8-10]. While cytoxicity is the focus of much current research in nanoscience, the mathematical modelling of nanoparticle cytotoxicity at the cell population level appears to have received little attention to date. Maher *et al.*[11], building on the work of Mukherjee and Byrne[12], have developed a model for the evolution of a population of cells by analysing the dependence of apoptosis on the loss of mitochondrial membrane potential.

Most of the models which have been developed for the uptake process have been at the cellular level, using models of the type described in the book by Lauffenburger and Linderman[13]. In general, these involve the development of systems of ordinary differential equations based on reaction schemes, beginning with an extracellular concentration of nanoparticles, which can attach to and detach from receptors on the cell surface, forming nanoparticle-receptor complexes. These complexes are then taken into the cell by endocytosis, forming endosomes. Some receptors may be recycled back to the cell surface from the endosomes[8]. Models of this type have been extended to incorporate flow cytometry data[14] and have also been specialized for particular types of nanoparticles, such as silicon quantum dots[15]. It has also been found that the process of cellular uptake of nanoparticles can be dependent on the cell cycle phase[16]. Åberg *et al.*[17] have developed an age-structured theoretical framework to model the dependence of nanoparticle uptake on the cell cycle.

Models of nanoparticle uptake at a population level have not received as much attention. Typically, it is assumed that cells uptake nanoparticles at a rate proportional to the difference between the rate of cell division and some maximum or limiting flux of nanoparticles. This leads to a simple exponential model for the concentration of nanoparticles inside the cells[9].

Results

Quantification of QDs

The FlowJo software package was employed to convert fluorescent emission from cells to relative amount of ingested nanoparticles. The gated consistent cell population with a narrow distribution profile was plotted in the FITC (green) or APC (red) fluorescence channel. In all samples, histograms showed a normal distribution. The scheme of subset selection and an example of population profiles are given in the Supplementary Information (Fig. 1 and 2).

Signals from cells in the control group (which did not receive any nanoparticles) were taken as baseline measurements. To estimate the percentage of the population which ingested quantum dots, histograms of the control and treated samples were overlaid. The overlap area was excluded from the analysis. The bright sub-population of cells which ingested QDs is shifted to the right. By integrating the shifted area, the percentage of cells which took up quantum dots can be found.

The intensity of uptake in the reference channel with respect to untreated cells can be semi-quantitatively described. The geometric mean was taken as the signal value; however, other statistic options (median or mean values) are also applicable, since the system behaves as a normal distribution.

Internalization of QDs

Flow cytometry allows the accurate collection of fluorescent signals which are quantized for each cell. Cells were grown in the presence of QDs for 12 and 24 hours, respectively. Control cultures

did not contain any nanoparticles. No deviation from the control was observed in cultures treated with 1-10 nM. Drastic changes were observed for samples exposed to 100 nM. The electron microscopy method (TEM) has been employed to visualize QDs intracellular localisation. The resulting images can be found in the Supplementary Information (Fig. 3).

Annexin V apoptosis test

The conditioned cell cultures were examined for prevalent cell fate. The Annexin V detection kit was employed to distinguish live, apoptotic and necrotic stages. The exposure to 1 or 10 nM of nanoparticles did not show any deviations from the control at any time point. No QD uptake was recorded. A drastic change was observed when monocytes exposed to 100 nM of QDs were tested. The amount of live, apoptotic (either in early and late stage) and necrotic cells greatly varied depending on QD parameters (size, composition).

Mathematical Modelling

The model is composed of five ordinary differential equations that describe the evolution of five distinct populations in time. These populations are the concentration of intracellular quantum dots $c(t)$, the number of cells in apoptosis $A(t)$, the number of cells in necrosis $N(t)$, the number of healthy cells $H(t)$, and the number of dead cells $D(t)$. The aim of the modelling is to quantitatively assess the response of a population of healthy cells to exposure from quantum dots. In particular, the model describes the transition of healthy cells to either a necrotic or an apoptotic state, and how the rate of these transitions depend on the intracellular quantum dot concentration $c(t)$. We begin by formulating an expression for $c(t)$.

The cells uptake the quantum dots via endocytosis. The endocytosis of nanoparticles is known to be a complex process, and has been shown to depend on such factors as the cell type, the shape and size of the nanoparticles, and the surface treatment the particles may have been subjected to[18-20]. We make the simple assumption here that the rate at which the cells ingest the quantum dots is

proportional to the difference between the saturation concentration of quantum dots in the cells, c_s, and the current intracellular concentration $c(t)$. The governing equation for $c(t)$ then takes the form

$$\frac{dc(t)}{dt} = k_c\big(c_s - c(t)\big),$$

where k_c is a rate constant. There are no quantum dots in the cells initially, so that $c(t = 0) = 0$. Solving the governing equation subject to the initial condition gives

$$c(t) = c_s(1 - e^{-k_c t}), \qquad (1)$$

so that $c(t)$ tends to the saturation concentration over a timescale determined by $1/k_c$. We should caution here that c_s (and perhaps even k_c) may depend on the initial extracellular concentration of quantum dots.

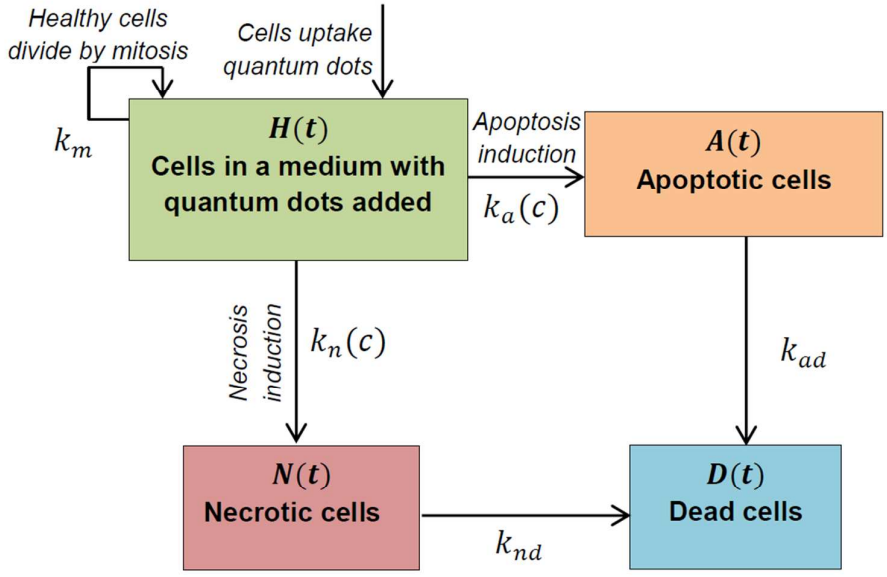


Figure 1: A schematic representation of the mathematical model. Each box represents a distinct subpopulation of cells whose evolution in time is tracked by the model. Cells uptake quantum dots via endocytosis, and the toxic effects of the QDs induce some of the cells to enter a necrotic or apoptotic state. These cells subsequently die. The rate at which the cells enter necrosis or apoptosis

depends on the intracellular concentration of quantum dots, which is why some of the rate constants above are shown to depend on c.

It should be emphasised that our model for the intracellular concentration of quantum dots omits many of the details of cellular quantum dot trafficking. For example, it does not distinguish between quantum dot subpopulations in endosomal and lysosomal compartments, and does not directly take account of binding to surface receptors. More sophisticated models for nanoparticle uptake and trafficking have in fact been proposed, examples being provided in the studies by Salvati *et al.*[9] and Wilhelm *et al.*[21] However, in our view there is not much point to developing more sophisticated models in the current context since the experimental data to hand is not sufficiently refined to distinguish which compartment the quantum dots are in.

We now turn our attention to the construction of the model equations for the four cell populations. **Figure 1** schematically represents the relationships between the cell populations and introduces some notation. Consider, for example, $A(t)$, the number of cells in apoptosis at time t. The rate of change of $A(t)$ is assumed to take the form

$$\frac{dA(t)}{dt} = k_a(c)H(t) - k_{ad}A(t), \qquad (2)$$

where the first term on the right hand side of (2) describes healthy cells entering apoptosis, and the second term describes the death of cells in apoptosis. Here $k_a(c)$ and k_{ad} are rate parameters and $k_a(c)$ is concentration dependent. It is natural here to take $k_a(c)$ to be a function of the intracellular quantum dot concentration c since we expect that the rate at which cells enter the apoptotic state should increase as the intracellular quantum dot concentration increases. However, once the cell has entered an apoptotic state, it is reasonable to assume that the rate at which the then cell progresses through apoptosis and dies is determined by the time scales of natural cellular processes, and so we take k_{ad} to be independent of c.

The equations for the other cell populations are constructed similarly, and read as follows

$$\frac{dN(t)}{dt} = k_n(c)H(t) - k_{nd}N(t), \quad (3)$$

$$\frac{dH(t)}{dt} = \big(k_m - k_a(c) - k_n(c)\big)H(t), \quad (4)$$

$$\frac{dD(t)}{dt} = k_{ad}A(t) + k_{nd}N(t). \quad (5)$$

Here the parameters characterising the rate of entry and exit from necrosis are given by $k_n(c)$ and k_{nd}, respectively. The reasoning for taking $k_n(c)$ to be dependent on c and for choosing k_{nd} to be independent of c is similar to that given above for the apoptosis parameters. For simplicity, we assume a linear relationship between $k_a(c)$, $k_n(c)$ and c, so that

$$k_a(c) = k_{a0} + k_{a1}c, \quad k_n(c) = k_{n0} + k_{n1}c \quad (6)$$

where $k_{a0}, k_{a1}, k_{n0}, k_{n1}$ are constants. We also assume that the rate of cell division is not affected by the presence of quantum dots, so that k_m does not depend on c. Furthermore, apoptosis and necrosis were observed to be negligible in the control experiments, so we take $k_{a0} = k_{n0} = 0$. Taking these assumptions into consideration, we rewrite Equations (2) – (5) as follows

$$\frac{dA(t)}{dt} = k_{a1}c_s(1 - e^{-k_c t})H(t) - k_{ad}A(t), \quad (7)$$

$$\frac{dN(t)}{dt} = k_{n1}c_s(1 - e^{-k_c t})H(t) - k_{nd}N(t), \quad (8)$$

$$\frac{dH(t)}{dt} = \Big(k_m - c_s(k_{a1} + k_{n1})(1 - e^{-k_c t})\Big)H(t), \quad (9)$$

$$\frac{dD(t)}{dt} = k_{ad}A(t) + k_{nd}N(t). \quad (10)$$

We solve the governing equations subject to the initial conditions

$$A(t=0) = 0, N(t=0) = 0, H(t=0) = H_0, D(t=0) = 0, \quad (11)$$

where here $t = 0$ corresponds to the time the cells were initially seeded in the culture, and H_0 is a constant representing the initial population of healthy cells. Note that (11) assumes that all of the cells are initially healthy.

The mathematical model is now developed and consists of equations (1) and (7) – (11). It is worth making a few brief comments about the structure of these governing equations. Assuming the rate parameters and the initial value H_0 are all known, equation (9) implies that $H(t)$ can be solved for independently of $A(t)$, $N(t)$, and $D(t)$. The independence of *H(t)* from the other cell population sizes is a consequence (principally) of our assumption that necrosis and apoptosis are irreversible processes - that is, once a cell enters a necrotic or apoptotic state, it cannot revert to a healthy state. We are also assuming here that competition for resources between the different cell populations is not a significant factor in the experiments. With $H(t)$ in hand, it can be used as data in equations (7) and (8), which may now be solved for $A(t)$ and $N(t)$, respectively. With $A(t)$ and $N(t)$ available, equation (10) may be integrated directly to give $D(t)$. Although considerable analytical progress can be made with the solution of these equations, the expressions arising are somewhat unwieldy, and so we prefer to take a numerical approach here.

Values for the parameters used in the model are given in Table 1. As the value for the saturation concentration c_s is not determined by the experiments, we combine this unknown parameter with k_{a1} and k_{n1} since the c_s term only appears in the model equations when combined with one of k_{a1} or k_{n1}. The parameter values shown were obtained using parameter estimation software provided by the simulation package COPASI. COPASI is an open-source (http://www.copasi.org) software application for the mathematical modelling and simulation of biochemical networks. It can be used to model both deterministic and stochastic systems, and provides a suite of numerical and analytical tools with which to analyse the governing equations arising. In particular, it offers a range of optimization methods for parameter estimation. Here, the method of Hooke & Jeeves, a direct

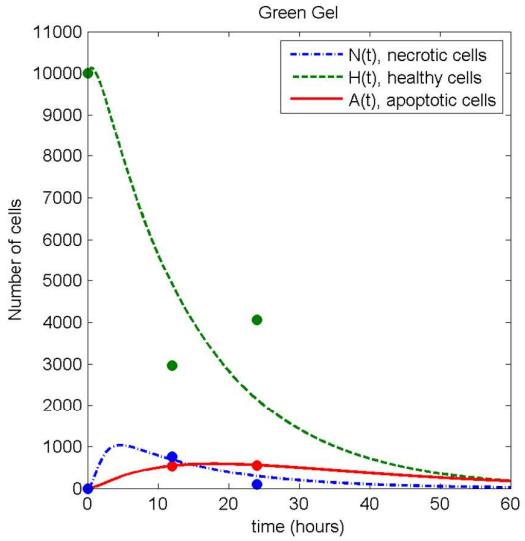
search algorithm, was used to obtain suitable parameter values. A good discussion of COPASI and its capabilities can be found in the paper by Hoops *et al.*[22] The software estimated the parameter values using the available experimental dataset.

Type	k_c	k_m	$c_s k_{a1}$	$c_s k_{n1}$	k_{ad}	k_{nd}
Units	hr^{-1}	hr^{-1}	hr^{-1}	hr^{-1}	hr^{-1}	hr^{-1}
Green Gel	1	0.05	0.009	0.11	0.05	0.86
Green TGA	0.81	0.12	0.3	0.06	0.14	0.1
Red Gel	1	0.05	0.148	0.07	0.04	0.48
Red TGA	0.167	0.05	0.144	0.1	0.04	0.76

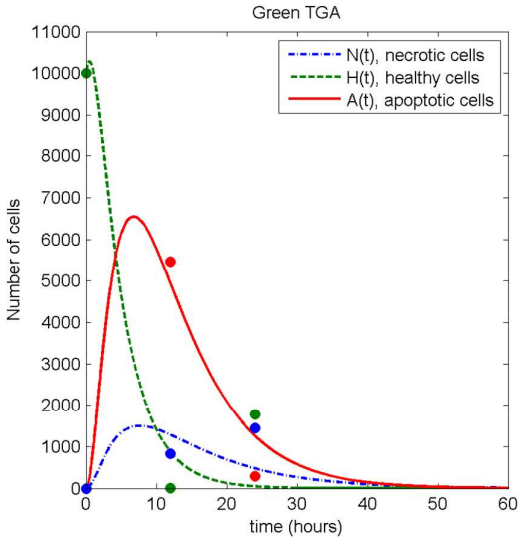
Table 1: Parameter values for four types of nanoparticles: green TGA-gelatin covered quantum dots (Green Gel); green TGA covered quantum dots (Green TGA); red TGA-gelatin covered quantum dots (Red Gel); and red TGA covered quantum dots (Red TGA)

Model results

The numerical results are displayed in Figure 2, and the parameter values used to generate these plots are listed in Table 1. For clarity, the evolution of the number of dead cells $D(t)$ is not displayed here. It is clear from these results that cells that enter necrosis die more rapidly than those that enter apoptosis. We also deduce that out of the four different types of QDs assessed, the green TGA-gelatin covered QDs are the least toxic to cells. The toxicity of the other three types of QDs seems to be comparable. Also, for an extracellular QD concentration of 100 nM, it is seen in all cases that the exposed cells die on a time scale of the order of days. It is clear from Figure 2 that for a number of the data points, there is a substantial discrepancy between theory and experiment. Unfortunately for these points, the experimental error bars arising are typically substantially smaller than the discrepancy between theory and experiment. This indicates that there may be aspects of the behaviour that the current model is failing to accurately capture in all cases.



A



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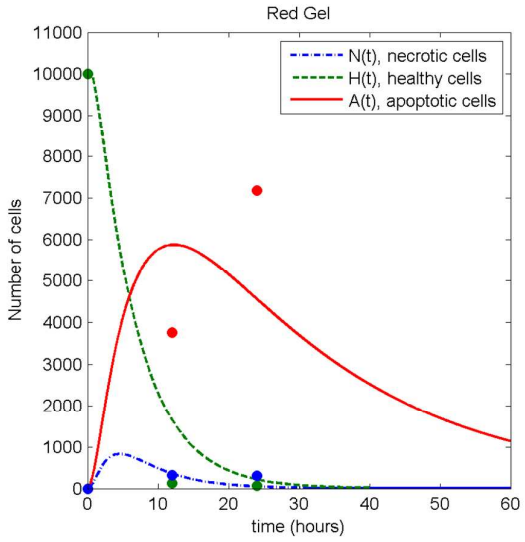
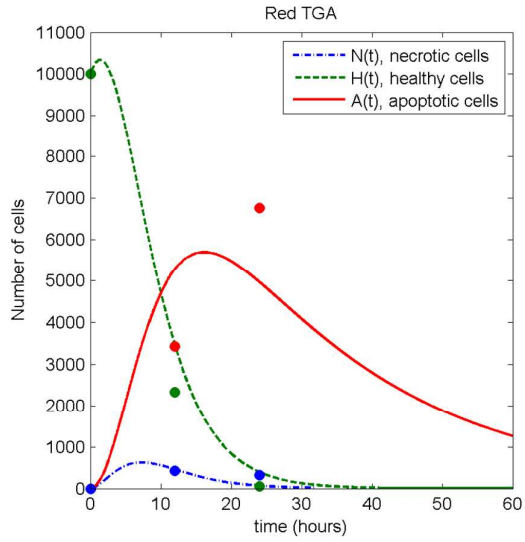


C



D

Figure 2: Evolution of RAW264.7 cells incubated with various types of 100 nM QDs over time. The composition of QDs is as follows. **A:** Green TGA-gelatin covered. **B:** Green TGA-capped. **C:** Red TGA-gelatin covered. **D:** Red TGA-capped

Discussion

The issue of QD cytotoxicity is a major obstacle for the clinical application of fluorescent nanoparticles. Their behaviour in physiological media is hard to predict due to the large number of factors which influence QD-cell interaction. Despite the wide range of existing experimental assays, most studies do not allow simultaneous quantification of intracellular QDs and evaluation of their effect on cell function; often multi-parameter methods are costly or time consuming. Techniques which explore QD fluorescence offer a large number of tests; however, it should be noted that the fluorescent signals of nanoparticles change after interaction with cellular compartments, and thus the QDs which are added to the culture and observed hours later have different properties. Sometimes experimental results do not give a clear answer, as they strongly depend on the complexity and relevance of the chosen biological model system. Taken together, mathematical modelling is a valuable tool in the analysis of experimental results.

In this paper, we have developed one of the first mathematical models to describe the toxic effects of quantum dots on cell populations. The model consists of a system of ordinary differential equations that tracks the evolution over time of four cell subpopulations, namely: healthy cells, apoptotic cells, necrotic cells, and dead cells. A simple model describing the cellular uptake of quantum dots is also considered. The relationship between the health of the cells and the presence of quantum dots is modelled by assuming a linear relationship between the intracellular concentration of quantum dots and rate parameters characterising the transition of healthy cells to an apoptotic or necrotic state. Model parameters were estimated by comparing the model output to flow cytometry data, and reasonable agreement between theory and experiment was found in all cases.

The model we have developed here has the advantage of being relatively simple and easy to use, with the toxic effects of the quantum dots being conveniently characterised by the values of just a

handful of parameters that can be estimated using experimental data. The model has a generic character. Although the uptake model is quite simple, it is likely to be capable of capturing the broad features of the uptake process of many nanoparticle/cell systems. Also, our modelling of toxin-induced necrosis and apoptosis is one of the simplest possible conceivable, and in our view provides a sensible starting point for the modelling of these processes. We are confident that the overall structure of this aspect of the model will be capable of modelling toxin induced necrosis and apoptosis in numerous nanoparticle/cell systems.

However, it should be conceded that there is considerable scope for improving upon the model. One weakness of the work described here is that no direct measurements of intracellular quantum dot concentrations were made, and this necessitated the use of a very simple model for quantum dot uptake. Measurements of intracellular quantum dot concentrations over time may allow for the development of a more accurate uptake model for a particular quantum dot/cell system. Also, the experimental data quantifying the various cell subpopulations was only available for three time points (t = 0 hrs, 12 hrs, 24 hrs), which is not sufficient to allow for accurate parameter estimation. More refined experimental data is also required to determine if the relationship between the intracellular quantum dot concentration and some of the model rate parameters is truly linear.

The model described can also be extended in a number of directions. One obvious extension is to incorporate more compartments in the model – for example, a model could easily be developed that has separate compartments for cells in early-stage and late-stage apoptosis. Another possibility is to develop an age-structured model that takes account of the stage of the cell cycle the cells are in. Such a model would be of particular value if multi-parametric flow cytometry data were available that was capable of simultaneously resolving the age and health of the cells.

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Materials and Methods

Experimental setup

Cell culture

The RAW 264.7 murine macrophage cell line was used in this study. Cells were cultured in Dulbecco's Modified Eagle Media (DMEM; Sigma), supplemented with 10% Foetal Bovine Serum (FBS; Sigma), 100 µg/mL of penicillin and 100 µg/mL of streptomycin. Macrophages were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

Annexin V Apoptosis Assay

In this assay, cells were seeded to a density of $2.5x10^5$ cells per well in 6 well-plates. After 24 hours of culture, appropriate amounts of QDs were added to each well. Control samples remained untreated. Cells were co-incubated with or without nanoparticles for 12 or 24 hours. Samples were harvested on the day of analysis. Cells were washed twice with PBS immediately after harvesting, re-suspended in a 500 µl buffer and stained with viability dye according to protocol. Afterwards cells were washed with serum-containing buffer. Finally, cells were prepared and stained with Annexin V Apoptosis Assay Kit (eBioscience) and directly proceeded to flow cytometry. All measurements were performed on BD FACS Canto A fitted with 2 lasers (blue, 488 nm; red, 633

nm) and 6 available colours. Unstained cells, single-stained samples, and cells treated with QDs only (without further staining) were used as quality controls.

Quantification of QDs

The amount of ingested nanoparticles was ascertained using the FlowJo software package. At least 10,000 events were recorded per tube. A consistent macrophage population was identified from the light scatter graph. The level of fluorescence in the FITC channel was evaluated from a histogram plot; the geometric mean value was used quantitatively as a statistical parameter. The percentage of population of interest was found from the overlay of two histograms of cells treated with QDs and untreated controls in the reference channel.

Mathematical model

In order to develop a mathematical framework for this scenario, it is necessary to make some basic assumptions, as follows. All cells are initially taken to be healthy. They may then enter an apoptotic or necrotic state, resulting in cell death. Thus, we introduce a compartment model consisting of four compartments, one for each state that the cell may be in, with associated transition rates between each compartment. We also assume that the transition rates depend on the concentration of quantum dots which the cells are exposed to, so it is necessary to develop an expression describing the intracellular concentration of nanoparticles. The resulting model equations are presented in Section 2.4, together with parameter values estimated by comparison of numerical simulations with the available experimental data. The model equations were solved numerically using the MATLAB package ode45. The COPASI software package was used to estimate the parameter values using the experimental dataset.

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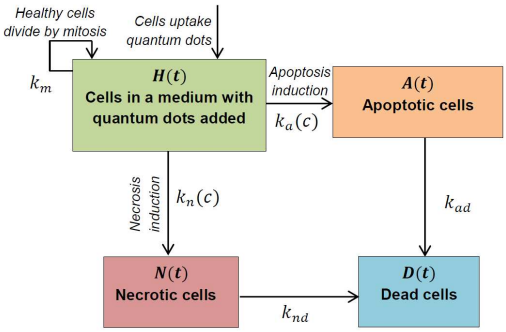

Scheme represents the model approach. After exposure to quantum dots initial cell population may follow various pathways which depend on intracellular concentration of quantum dots