<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>Quantum dots in bio-imaging and drug delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Gladkovskaya, Olga</td>
</tr>
<tr>
<td><strong>Publication Date</strong></td>
<td>2015-11-23</td>
</tr>
<tr>
<td><strong>Item record</strong></td>
<td><a href="http://hdl.handle.net/10379/5448">http://hdl.handle.net/10379/5448</a></td>
</tr>
</tbody>
</table>
Quantum Dots in Bio-imaging and Drug Delivery

By Olga Gladkovskaya

A thesis submitted to the College of Science
National University of Ireland, Galway
In partial fulfilment of the requirements for the degree of

Doctor of Philosophy

National Centre for Laser Applications
School of Physics
College of Science
National University of Ireland, Galway

Academic Supervisors
Dr. Yury Rochev
Dr. Gerard M. O’Connor
# CHAPTER 1: INTRODUCTION

## 1.1 BACKGROUND

1.1.1 Quantum confinement  
1.1.2 Optical properties  
1.1.3 Intracellular fate  
1.1.4 Surface chemistry  
Ligand exchange  
Silica incorporation  
Micelle growth

## 1.2 QD UPTAKE BY LIVE CELLS

1.2.1 Endocytosis mechanisms  
1.2.2 Methods of investigation  
Energy inhibition  
Pharmacological inhibitors  
Expression of mutated proteins and use siRNA

1.2.3 Mechanism of QD uptake

## 1.3 QD IN VITRO CYTOTOXICITY

1.3.1 Factors influencing QD cytotoxicity  
1.3.2 Mechanisms of cytotoxicity  
ROS formation  
Apoptosis  
Autophagy  
Necrosis  
1.3.4 Cytotoxicity of QD bio-conjugates

## 1.4 IMMUNE RESPONSES CAUSED BY NANOPARTICLES

1.4.1 Carbon black (CB) nanoparticles  
1.4.2 Silica nanoparticles  
1.4.3 TiO₂ nanoparticles  
1.4.4 Silver and Gold nanoparticles (Ag and Au NPs)  
1.4.5 Iron-containing nanoparticles (Fe NPs)  
1.4.6 Cerium oxide nanoparticles

1.4.7 Conclusion

## 1.5 TERNARY QANTUM DOTS

1.5.1 Benefits and prospective  
1.5.2 Antimony sulpho-iodide

## 1.6 METHODS OF QD QUANTIFICATION

1.6.1 Light microscopy  
1.6.2 Flow cytometry  
1.6.3 Electron microscopy  
1.6.4 Mass spectrometry  
1.6.5 Mass cytometry

## 1.7 CONCLUSION

OBJECTIVES

# CHAPTER 2. MATERIALS AND METHODS

## 2.1 CHEMICAL METHODS

2.1.1 Quantum Dots synthesis
Materials 50
Method 50
UV-vis and PL spectra 52
Particles further storage and use 53
2.1.2 SbSI SYNTHESES AND MODIFICATION 53
Standard synthesis procedure 53
Powder X-ray diffraction (XRD) structural analysis 53
Electron microscopy 54
Treatment with high power ultrasound 54
Nano-needles optical properties test 54
2.2 CELL CULTURE 54
Materials 54
2.2.1 CELL LINES 55
RAW264.7 55
MS-5 and 3T3 cell lines 55
U937 cell line 56
2.2.2 PRIMARY CELLS 56
Periphery blood mononuclear cells (PBMCs) extraction and maintenance 56
Cell growth study on cardiovascular stents 57
2.3 ELECTRON MICROSCOPY 57
2.3.1 TRANSMISSION ELECTRON MICROSCOPY (TEM) 57
Cell culture 57
Primary fixation 57
Secondary fixation 58
Dehydration 58
Resin embedding 58
Cells sections and imaging 58
2.3.2 SCANNING ELECTRON MICROSCOPY (SEM) 59
Fixation 59
Dehydration 59
Drying 59
2.4 BIOLOGICAL IN VITRO ASSAYS 60
2.4.1 LIVE/DEAD ASSAY 60
2.4.2 FLUORESCENT MICROSCOPY 60
2.4.3 ALAMAR BLUE 60
2.4.4 DS-DNA QUANTIFICATION 61
2.5 QDS UPTAKE STUDY BY FLOW CYTOMETRY 61
2.5.1 PRINCIPLES OF THE METHOD 61
2.5.2 SAMPLE PREPARATION FOR FLOW CYTOMETRY 62
2.5.3 GATING STRATEGY 62
2.5.4 ANNEXIN V APOPTOSIS ASSAY 63
2.5.5 QDS UPTAKE AND CD80/86 SURFACE MARKERS EXPRESSION 64
2.5.6 INTRACELLULAR QUANTIFICATION OF QDS 65
2.6 CONCLUSION 65

CHAPTER 3. OVERVIEW 66
CONTRIBUTION TO THE PUBLISHED PAPERS 72
REFERENCES 74

CHAPTER 4. PUBLISHED PEER-REVIEWED PAPERS 85

CHAPTER 5: CONCLUSION 138
Declaration

The work in this thesis is based on the research carried out at the National Centre for Biomedical Engineering (NCBES) and National Centre for Laser Ablation (NCLA), School of Physics, National University of Ireland Galway. I, Olga Gladkovskaya, hereby certify that this thesis has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a degree or qualification.
Acknowledgments

First and foremost I’d like to say big thanks to my supervisors Dr. Yury Rochev and Dr. Gerard O’Connor for their endless patience and attention. Can’t say enough how much I’m grateful for believing in me and giving the chance. Thanks for all effort you put in bringing me here. It’s a great honour to be part of your team.

Also I want to express my gratitude to present and former NCBES and NCLA staff, Dr. Muriel Voisin, Hanno Conring, Dr. Shirley Hanley, Liam Brennan for tremendous help and expertise. There’s special acknowledgement to Rebecca Nolan for priceless day-to-day assistance in every query. Thanks, Rebecca, for being with me all these years!

Another person worth to be mentioned separately is Clare McDaniel. We’ve been all tough times together, don’t know how I’d do it without you.

Next, I’d like to thank truly inspirational people for sharing their knowledge and charisma: Prof. Yury Gun’ko, Dr. John Faul, Dr. Mikhail Nosov, Prof. Rhodri Ceredig, Dr. Elena Voloshina, prof. Beate Paulus. I’ve learnt so many things from you.

It’d be impossible to go all this way without constant moral support from my family. I’m proud to have you on my side. And of course I have to mention my dear friends who always believed in me at moments when even I was close to give up. Radu Groza, Claudia Di Salvo, Sara Tabea Helm, Sarah Alsobegh, Dr. Sandeep and Vrushali Pande, Dr. Suraya Diaz, Gerard Caffrey, Dr. Vitaly Panov, Evgenij Grigoriev, Dimitri Ciornii – you’re fantastic people.
List of abbreviations

3-MA - 3-Methyladenine
Ac-DEVD-cho - N-acetyl-L-α-aspartyl-L-α-glutamyl-N-(2-carboxyl-1-formylethyl)-L-valinamide
AO – atomic orbital
Apafl - Apoptotic protease activating factor 1
APC – Allophycocyanin
ATP - adenosine triphosphate
AUT - amino undecanethiol
BAL - bronchialveolar lavage
Beta-CD/GA - beta-cyclodextrin/glycirrhizic acid
Bax – Bcl2 associated X protein
Bcl2 – B-cell lymphoma 2
Bcl-xl – B-cell lymphoma extra-large
BID – Bcl2 interacting protein
C. jejuni - Campylobacter jejuni
cAg - colloidal silver (Ag)
CB – carbon black
CBNPs – carbon black nanoparticles
CCL – chemokine C-C motif ligand
Cdc42 - Cell division control protein 42 homolog
CIE – clathrin-independent endocytosis
CME - clathrin-mediated endocytosis
COX – cyclooxygenase
CPD – critical point drying
CXCL – chemokine C-X-C motif ligand
CYST – cysteamine
DNA – deoxyribonucleic acid
DOS – density of states
ds-DNA – double-stranded DNA
E. coli - Escherichia coli
E. faecalis - Enterococcus faecalis
ER – endoplasmic reticulum
ERK – extracellular signal-regulated kinase
EtOH – ethanol
FACS – fluorescent activated cell sorting
FADD - Fas-Associated protein with death domain
FBS – fetal bovine serum
FITC - fluorescein isothiocyanate
FRET - Förster resonance energy transfer
FWHM – full width of half maxima
GFP – green fluorescent protein
GSH – glutathione
GSSG – glutathione disulfide
GTP - guanosine triphosphate
HBSS - Hank's Balanced Salt Solution
HEK - human embryonic kidney
HMMs – human monocyte-macrophages
HOMO – highest occupied molecular orbital
HSP – heat shock protein
HUVEC - Human Umbilical Vein Endothelial Cells
ICP-AES - inductively coupled plasma atomic emission microscopy
ICP-MS - inductively coupled plasma mass spectrometry
IFN-γ – Interferon gamma
IgE - Immunoglobulin E
IL – interleukin
iNOS – isoform nitric oxide synthase
LC3-I/II – light chain protein (form I or II)
LDH – lactose dehydrogenase
LMP – lysosome membrane potential
LPS – lipopolysaccharide
LUMO – lowest unoccupied molecular orbital
MAA - mercaptoaetic acid
MCA – methacrylic acid
MCP-1 - Monocyte chemoattractant protein-1
MIP-2 - macrophage inflammatory protein-2
MMP – mitochondria membrane potential
SEM – scanning electron microscopy
siRNA – small interfering RNA
Smac/Diablo - second mitochondria-derived activator of caspases/ Direct IAP-Binding protein with Low PI
SOD - superoxide dismutase enzyme
STED - Stimulated emission depletion
tBID – truncated form of BID protein
TEM – transmission electron microscopy
TGA – thioglycolic acid
Th1(2) – T-helper cell type 1 (2)
TLR – Toll-like receptor
TNF-a – tumour necrosis factor alpha
TOPO – trioctylphosphine oxide
TRADD - Tumor necrosis factor receptor type 1-associated death domain protein
UV – ultra violet
XRD – X-ray diffraction
zIETD-fmk - Z-Ile-Glu(OMe)-Thr-Asp(OMe) fluoromethylketone
zVAD-fmk - Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone
Abstract

This work explores a range of bionanomaterials, with particular attention to their influence on cell growth, function and fate. Also the issue of intracellular nanoparticles quantification has been addressed in experimental and model approach. Ternary V-VI-VII semiconductor material SbSI has been investigated for its capacity to replace existing convenient II-VI CdTe, which is commonly used for engineered Quantum Dots (QDs).

QDs are typically composed of heavy metals, and so cytotoxicity continues to be a major concern. It remains unclear how QD uptake and cell fate are influenced by QD parameters such as size, composition, coating, concentration, and time of exposure. To help elucidate such issues, we have investigated experimentally and theoretically the toxic effects of intracellular QDs on RAW264.7 cell line. We have performed comprehensive study of QDs uptake dynamics and kinetics by monocyte/macrophage cells in physiological media conditions depending on particles size, composition, concentration, and exposure time using flow cytometry as quantification method. Number of cellular and immune responses was measured at the same time by multicolor approach. Has been measured the distribution of live, necrotic, early and late apoptotic cells under different experimental conditions. Pro-inflammatory surface markers CD80/86 were profiled as macrophage activation parameter. Flow cytometry has been shown as quick unbiased evaluation method of ingested particles on population level. We explained sudden drop in surface markers expression related to QDs uptake and cell function profile. The model parameters enabled a convenient quantitative evaluation of the toxicity of the various types of QDs, and good agreement was found between theoretical and experimental results. We have developed water-based ultrasonic synthesis of micro-scale SbSI particles to bring it down to nano-scale level. We investigated on reaction mechanism and revealed the role of intermediate product – antimony trisulfide. Also was made successful attempt to control crystal growth of particles with various dimensions were obtained. As-prepared micro- and sub-micro crystals were broken down to nanoparticles with broad absorption. The potential cytotoxicity of different fractions was tested on RAW264.7 monocytes.
Graphical abstract

Part I

- Explore potential of QDs in bio-imaging and drug delivery
  - Development of simultaneous QD quantification and cellular response acquisition method
  - Synthesis and bio-assays of CdTe QD alternate material
  - Study of cell fate by flow cytometry
  - Microscopy imaging: TEM, fluorescent, light and confocal
  - Development of mathematical model for CdTe QD cytotoxicity

Part II

- Exposure to CdTe QDs (2.5 nm)
- Cell growth study on laser rippled stainless steel and platinum-chromium surface (29-55 nm roughness)
- Study of cellular responses to different nanomaterials
- Exposure to boron nitride nanotubes (15 nm thickness)
- Exposure to SbSI sub-micro needles (100 nm thickness)
Chapter 1 : Introduction
1.1 Background

The field of nanoscience is rapidly developing, due to the discovery of exclusive features of nano-sized matter, which showed intermediate versatile properties between single molecules and bulk solids. Therefore, it has become possible to find smart solutions for a number of challenging tasks, such as alternative energy sources [1-3], high-effective catalysis [4, 5], construction of new generation of electronic devices [6-8], fabrication of high-conductivity elements useful in printed electronics [9], controllable drug release [10-13], cancer diagnostics and labeling [14], easy and fast chemical, biological and medical assays [15]. The materials used in nanoscience must be stable to media conditions, resistant to alterations, cheap in manufacturing, easy in use and non-hazardous. Thus, the challenge to design materials to relevant conditions is a strong consideration.

Quantum dots (QDs) were discovered by M. A. Reed in 1985. [16]. They're usually defined as inorganic nano-scale crystals with a number of unique physical and chemical size-dependent properties. In comparison to current fluorophores (organic dyes and lanthanide chelates), QDs demonstrate better characteristics, such as narrow absorption and emission peaks (Fig.1.1), prolonged energy separation between absorption and emitting spectra, slower decay radiative life time, better resistance to photobleaching, higher brightness and fluorescent time, possibility of excitation by non-specific wavelength (even in NIR spectra), which makes them attractive agents for bio-imaging. Unlike organic dyes, QDs properties can be adjusted by core size and composition, coating material and thickness variation, depending on the eligible application. In contrast to molecular fluorophores, QD nanocrystals are suitable for multi-color imaging, tunable by means of their size. The common material for fabrication is II-VI or III-V semiconductors, like as CdSe, CdTe (commercially available products), GaP, InP [17], AsP etc. Their composition ranges from a few hundreds to thousand atoms and therefore, significant number of atoms are exposed to surface. Consequently, the nanoparticles have lots of dangling bonds at the surface, which causes exceptional reactivity. In fact, this feature leads to decreasing biocompatibility [18], due to the essential risk of releasing ions from the particle and triggering the cell's apoptosis. As a result, rapid surface degradation excludes implementing bare core QDs in vivo and in vitro applications [19]. Furthermore, the degradation affects optical properties of the
QDs. This results in emission spectra irregularities, which leads to irreproducible results. Thus, QDs need to be isolated, in order to increase quantum yield. Typical QDs have the core-shell structure. Many optical properties depend on particles composition, e.g. material what they're made from: II-VI (CdTe, CdSe), III-V – InP [17] or ternary semiconductors, like InGaP [20, 21]. These materials are alternatives to the Cd containing compounds. However, in spite of wide range of existing combinations, the most popular materials still remain CdTe and CdSe because of ease in synthesis, characterisation, functionalising and broad application. Another essential factor influencing their properties is size. The quantity of atoms exposed to surface significantly increases as the diameter is reduced.

In order to fix this issue encapsulation methods are in widespread use. For these purposes ZnS [10, 22], silica [23], CdS [22, 24], gelatin [25] and albumin are commonly used. Adding the passivation layer is observed as a red shift in absorption and emission [26]. The diameter is also increased. The next step in creating nanoparticles with eligible characteristics is by grafting to different ligands. Each type of ligand determines the specific application. The most common are poly(ethylene) glycol - PEG [13, 26], antibodies, antigenes, peptides [27], trioctylphosphine oxide - TOPO [28], methacrylic acid - MCA [13], mercaptoacetic acid – MAA [29]. And finally, functional biomolecules should be attached, mostly drugs [10, 11] or signaling peptides [29]. In summary, QDs gained significant results in fluorescent bio-imaging and detecting as broadly used agents and in the future, within the search and development of new materials [30], they have all chances to become part of personalized drug delivery systems [12, 13], cancer diagnostics and treatment [18].

1.1.1 Quantum confinement

Quantum Dots possess unique set of optical properties due to their intermediate state between single molecule and bulk material. Thus QD can be considered as combination of both. In a semiconductor upon light absorption electron gets promoted from valence band to conduction band; this leaves a hole behind. Both of them, electron and hole, form a unit called exciton. There’re two different types of exciton: Mott-Wannier and Frenkel. Semiconductors have Mott-Wannier exciton due to large dielectric constant. This type of exciton has weak Coulomb interaction because of big electron-hole distance. Frenkel
exciton is typical for organic compounds, due to close proximity of electron and hole. Wave-like properties of electron and hole define optical and electrical properties of nanostructures [31].

Quantum confinement takes place when size of the object is comparable to the wavelength of exciton. A QD is zero-dimensional relative to bulk; limited number of excitons results in quantized discrete energies in density of states (DOS). The step-size between energy levels is proportional to the reciprocal to QD radius.

Practically, quantum confinement in QD leads to blue-shift (broadening) of band-gap energy and its value depends on semiconductor type. This effect also allows to regulate band gap width by QD size [32]. Due to direct relationship between particle diameter and band gap energy, it's simple to generate series of differently bright colored QDs. Compared to bulk material, QDs own discrete energy levels due to small number of atoms in their core. As a consequence, each of these levels can be described by wave functions similar to atoms. Thus, QDs emission profile is very narrow and sharp, close to atomic-like.

The band gap is often defined as energy needed to create an electron and a hole at equilibrium state, when kinetic energy is equal to zero, at a distance sufficient to neglect Coloumbic interaction [31]. An exciton is coupled electron-hole pair with energy slightly lower than band gap. The exciton is often called a quasiparticle and behaves likewise hydrogen atom; the only difference is that instead of proton, a nucleus is formed by a hole. Thus, we can describe an exciton by Schrödenger's equation. However, it should be taken in consideration, that the hole mass is significantly less than proton's. In bulk semiconductor crystal the size of the exciton is characterized by Bohr radius \( r_B \) and defined by equation, where \( \varepsilon \) - the optical dielectric constant, \( h \) - reduced Planck's constant, \( e \) - the charge of an electron, \( m_e \) and \( m_h \) are effective masses of electron and hole, respectively.

\[
r_B = \frac{\hbar^2 \varepsilon}{e^2} \left( \frac{1}{m_e} + \frac{1}{m_h} \right)
\]

If the radius of the nanoparticle is comparable to the Bohr radius or smaller, the motion of the electron and holes are confined spatially [33-35] to the QD dimension which increases the transition energy of exciton and resulted in photoluminescence and blue shift.
1.1.2 Optical properties

The exceptional QDs characteristics make them a very prominent tool in optical biological imaging. Classical bio-imaging is based on organic dyes attached to a biological species to illuminate the target. Molecular fluorochromes are extensively used as fluorescent reporters in various bioconjugates, such as antibodies and molecular beacons. However, this class of dyes suffers from number of disadvantages:

1. High photosensitivity. It results in short time when assay has to be performed. Besides that, during whole preparation procedure, the sample must be protected from the light.
2. Narrow absorption. Most conventional fluorophores require excitation at a specific wavelength, which is not possible in many cases (e.g. if lasers are source of excitation) and limits number of dyes which can be excited by the single source.
3. Broad emission bands. Extended wavelength tails in the emission profile result in spectral overlap if multiple stains used. This makes it more difficult to interpret the signal, and demands use of extra controls to compensate signal spillover between channels.
4. Sensitivity to media conditions. The signal from fluorophore undergoes drastic degradation if pH values of the media fluctuate. Some dyes work over a limited temperature range.

In this context, QDs are promising candidates to replace known fluorophores due to following beneficial properties:

1. High quantum yield. QDs demonstrate high fluorescence brightness.
2. Broad absorption spectrum. Multiple QDs can be excited by the same source of light.
3. Narrow emission. Have sharp symmetric emission profile; several QD colors can be used in the same probe.
4. Large Stokes shift between maximum of absorption and emission wavelength.
5. Ease in surface functionalising. It enables attachment of different biomolecules for selective targeting regardless QD size.
6. Desirable emission/absorption wavelengths can be adjusted by QD size.
7. Resistance to photobleaching. Quantum dots do not fade when exposed to direct light.
8. Inert to environmental conditions. The nanoparticles don’t precipitate over long time if stored at 4°C.

9. NIR QDs can be used in clinical applications for non-invasive imaging, where autofluorescence from tissues can be avoided. Cells have high background fluorescence in green spectrum, that’s why imaging at far red region will provide clear picture without signal noise.

Fig. 1.1 depicts a typical QD absorption/emission profile vs. Rhodamine 6G. Within the same fluorescence intensity nanoparticles have more advantages in application over organic dyes. The main benefit of Cd-based QDs is their controllable size distribution via synthesis conditions, which enables to obtain particles with predictable absorption and emission wavelengths. It helps to avoid emission anisotropy and makes spectrum smooth and highly symmetrical. At the same time, the complicated decay behavior [36], surface chemistry, size effects and cytotoxicity limits their use. The scope of differences in physicochemical between molecular fluorochromes and QDs represented in Table 1.1

![Fig. 1.1](image_url) (a) absorption and (b) emission profiles of rhodamine 6G and CdSe QDs [37].

Table 1.1 Comparison of properties of organic dyes and QDs (based on information taken from [38])

<table>
<thead>
<tr>
<th>Property</th>
<th>Molecular fluorochrome</th>
<th>Quantum Dots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light absorption spectrum</td>
<td>Discrete bands, FWHM 35 nm to 80-100 nm</td>
<td>Smooth and broad; enables free selection of excitation wavelength</td>
</tr>
<tr>
<td>Molar absorption coefficient</td>
<td>2.5×10⁴-2.5×10⁵ M⁻¹ cm⁻¹ (at long-wavelength absorption)</td>
<td>10⁵-10⁶ M⁻¹ cm⁻¹ at first exitonic absorption peak,</td>
</tr>
<tr>
<td>Property</td>
<td>Description</td>
<td>Description</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Absorption at given wavelength</td>
<td>maximum)</td>
<td>increasing toward UV wavelengths; longer wavelength QDs generally have higher absorption</td>
</tr>
<tr>
<td>Emission spectra</td>
<td>Asymmetric, often tailing to long-wavelength side; FWHM, 35 nm to 70-100 nm</td>
<td>Symmetric, Gaussian profile; FWHM, 30-90 nm</td>
</tr>
<tr>
<td>Stokes shift</td>
<td>Normally &lt;50 nm, up to &gt;150 nm</td>
<td>Typically &lt;50 nm for visible wavelength-emitting QDs</td>
</tr>
<tr>
<td>Quantum yield</td>
<td>0.5-1.0 (visible), 0.05-0.25 (NIR)</td>
<td>0.1-0.8 (visible), 0.2-0.7 (NIR)</td>
</tr>
<tr>
<td>Fluorescence lifetime</td>
<td>1-10 ns, mono-exponential decay</td>
<td>10-100 ns, typically multi-exponential decay</td>
</tr>
<tr>
<td>Solubility or dispersibility</td>
<td>Control by substitution pattern</td>
<td>Control via surface chemistry (ligands)</td>
</tr>
<tr>
<td>Binding to biomolecules</td>
<td>Via functional groups; Often several dyes bind to a single biomolecule</td>
<td>Via ligand chemistry; Several biomolecules bind to a single QD</td>
</tr>
<tr>
<td></td>
<td>Labeling-induced effects on spectroscopic properties of reporter studied for many common dyes</td>
<td></td>
</tr>
<tr>
<td>Size (primary core diameter)</td>
<td>~0.5 nm</td>
<td>2-10 nm (core size)</td>
</tr>
<tr>
<td>Thermal stability</td>
<td>Dependent on dye class; can be precarious for NIR-wavelength dyes</td>
<td>Very stable; depends on shell or ligands</td>
</tr>
<tr>
<td>Photochemical stability</td>
<td>Sufficient for many applications (visible wavelength), but can be</td>
<td>Very stable (visible and NIR wavelengths); orders of magnitude higher than that of</td>
</tr>
<tr>
<td>Criteria</td>
<td>Characteristics</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Insufficient for high-light flux applications; often problematic for NIR-wavelength dyes</td>
<td>Organic dyes; can reveal photobrightening</td>
<td></td>
</tr>
<tr>
<td>Toxicity</td>
<td>From very low to high; dependent on dye; Considering in section 1.2 QDs <strong>in vitro cytotoxicity</strong></td>
<td></td>
</tr>
<tr>
<td>Reproducibility of labels (optical, chemical properties)</td>
<td>Good, owing to defined molecular structure and established methods of characterization; available from commercial sources; Limited by complex structure and surface chemistry; limited data available; few commercial systems available</td>
<td></td>
</tr>
<tr>
<td>Applicability to single-molecule analysis</td>
<td>Moderate; limited by photobleaching; Good; limited by blinking</td>
<td></td>
</tr>
<tr>
<td>Suitability for multi-color assay</td>
<td>Possible, restricted to 3-4 colours; Ideal for multi-color experiments; up to 5 colors demonstrated</td>
<td></td>
</tr>
</tbody>
</table>

### 1.1.3 Intracellular fate

There’s a great interest in QD ability to be ingested by live cells. It can be used for cell targeting, tracking and selective drug delivery. That’s why understanding of cell-QD interaction is of key importance. This includes investigation on mechanism of QD recognition, uptake, interaction with intracellular compartments, effect on cell function, and finally, fate of either QD and the cell.

Quantum dots are shown to be toxic to cells [39]. After crossing cellular membrane, nanoparticles start their journey in primary endosomes. Being trapped into vesicles, ingested nanoparticles are transported to different organelles during endocytosis. Those foreign bodies, which are not identified by the cell, are passed to lysosomes and peroxisomes [40]. These compartments have acidic and oxidative environment configured to destroy unrecognised species. It has been shown that such conditions resulted in the elimination of surface molecules. Introduction of QD bare core to the cell [41] causes release of Cd$^{2+}$ ions. Due to high surface-to-volume ratio, big number of atoms with the unsaturated bonds results in free radicals formation. Also QD
cytotoxicity depends on particle diameter – small green-emitting ones (approx. 2 nm) have greater number of surface atoms and easily penetrate the nucleus while larger red-emitting QDs (approx. 5 nm core) are less aggressive and remain within cytoplasm \[42\]. The transformation into toxic objects, as result of QD digestion by the cell, inevitably leads to a number of morphological and biochemical alterations. This includes cytoplasm granulation, loss of cell function, nucleus fragmenting, chromosome damage and eventually, cell death. In order to minimize the damage, appropriate QD composition, doses, exposure time and conditions must be used. Nevertheless, above mentioned parameters are varied for different cell types and animal models.

To reduce ineligible negative effects and to improve QD compatibility nanoparticles are incorporated into passivating shells, which can either be inert or have affinity to the cell. Numerous strategies have been explored, the most popular are covering by silica \[43-45\], packing into polymer micelles \[19, 46, 47\], decorating the surface with various biomolecules (proteins, aminoacids, mercaptoacids, carboxylic acids). At the same time, adding the extra layers significantly increases hydrodynamic size of particles and affects the endocytosis pathway.

1.1.4 Surface chemistry

The inherent property of QDs is big number of surface atoms with unsaturated bonds. It gives rise to their exceptional reactivity. This affects the physical and chemical stability of nanoparticles and results in emission spectrum irregularity and drop in QY. Apart from that, bare QDs are extremely aggressive to cells and cause lots of damage. Solution of these problems lies in packing up particles into multiple passivation and functional layers. As first line of defense, QDs are covered by semiconductor shell, which is typically not exceeding 1-2 monolayer and does not add significant thickness to the particle core. Capping material is usually chosen from semiconductors with minima lattice mismatch and the same structural type. This can be done during the synthesis or afterwards. The most common compounds are ZnS, CdS, ZnTe, ZnSe. This step helps to stabilize QD photoluminescence and prevent particles clumping and colloid sedimentation over time.

At early years, when the concept of QDs was under intensive development, synthesis in organic solution was the basic technique \[48\]. As-prepared nanocrystals had hydrophobic nature and were of no use in biological applications requiring aqueous
conditions. Consequently, solubilization is the next stage of post-synthetic treatment. Three main strategies are commonly used: ligand exchange, silica incorporation, micelle growth [49, 50]. Fig. 1.2 schematically shows mentioned ways.

**Ligand exchange**

The hydrophobic molecules stabilizing QD colloidal system during the synthesis can be substituted by bipolar compounds easy soluble in water. These molecules have both positively and negatively charged functional groups, one is attached to QD surface and oppositely charged one ensures hydrophilicity. The typical agents are aminoacids, carboxylic acids, mercaptoacids, thiols. Unfortunately, in many cases hydrophilic QDs showed reduce QY compared to their colloids in organic solvents [51, 52].

**Silica incorporation**

Alternatively, QDs can be solubilised by silanisation [43-45, 53]. The notable disadvantage of this method is QD size increase, as-modified particles range from tens nanometers [45] to few micrometers [53]. Wolcott et al in 2006 [44] has reported preparation of CdTe QDs prepared in one-pot synthesis with silica coating layer only 2-5 nm with final particles size less than 12 nm. Also this method is more complex than two others and suitable only for diluted systems. However, silica and its derivatives is the leading non-toxic biomaterial used routinely in biomedicine for numerous applications, including magnetic particles for MRI, drug delivery vectors, bioglasses for bone augmentation and periodontal repair, various implants and etc.
**Micelle growth**

Synthesized in organic solvents QDs possess higher quantum yield comparing to particles obtained in aqueous process. Incorporation into multilayered biosurfactant shell helps to solubilise nanoparticles with minimal loss in fluorescence. Typically, QDs in organic solvent phase (chloroform) injected into phospholipids or surfactants mixture containing various hydrophilic functional groups (-NH$_2$, -COOH, -PEG) [46, 49]. Also these groups provide sites for further engrafting to biomolecules. Under rigorous stirring and heating conditions, chloroform evaporates from biomolecules. Under rigorous stirring and heating conditions, chloroform evaporates from microemulsion and results in nanoparticles interfacial transfer. The main downside of micelles is significantly bigger particle diameter (>10 nm). Another approach is demonstrated by Chen and Rosenzweig in 2002 [54], who loaded a big siloxane micelle (over 100 nm) with multiple QDs. Afterwards, these core/shell water soluble nanoparticles receive their surface functionalisation. The options are almost unlimited and dictated only by final goal. It can be conjugating to antibodies, drugs, molecular beacons, proteins, receptors, DNA or RNA fragments and list goes on. They can be also incorporated into microspheres and hydrogels.

1.2 QD uptake by live cells

1.2.1 Endocytosis mechanisms

To sustain homeostasis, cells need to ingest nutrients necessary for stable and healthy functioning. While small species like small molecules (O$_2$, CO$_2$) can penetrate through membrane passively by concentration gradient, larger entities (e.g. proteins) have to be recognized by receptors and require active transport. Endocytosis is energy-dependent transfer of large molecules inside the cells. It can be used to regulate cell-cell signaling, receive essential compounds for life cycle maintenance, destroy potentially hazardous material. In the first approach, endocytosis can be divided into 2 types: phagocytosis (from greek “cell eating”) and pinocytosis (“cell drinking”). The purpose of phagocytosis is to trap and digest foreign bodies which were recognised as non-self and thus carrying a threat. Typically it targets bacteria and compartments larger than 500 nm. Special type of immune cells – phagocytes – are employed to execute the process;
they circulate with the blood stream or permanently reside in tissue and continuously scanning the environment. Monocytes/macrophages (either circulating in peripheral blood or residing in tissue) and dendritic cells (bone marrow) are professional phagocytic cell types.

Second type, pinocytosis, is aimed to engulf particles from the surrounding liquid media. There're several types of pinocytosis differ from each other by mediators, which, in turn, depend on target dimensions.

- **Caveolae** are small flask-shaped pits (approx. 50-80 nm) containing mainly protein caveolin. In endocytosis these membrane invaginations are designed to accommodate species (e.g. bacteria, viruses) in size range 200-500 nm entering the cell. However, endocytosis is not their primary function.

- **Clathrin-dependent** endocytosis is a most common pathway which accommodates intracellular trafficking of nutrients, growth factors, antigens, receptors. Clathrin-coated pits after engulfing intruders are fused with the early endosomes, which, in turn are appeared as sorting centre of the content. They also control activity and destination of taken up compounds.

- **Clathrin-independent (lipid raft).** These routes can deliver compounds to Golgi apparatus, endoplasmic reticulum (ER) and recycling endosomes. Clathrin-independent pathways are susceptible to cholesterol depletion, supporting the idea of lipid raft mechanism.

It's been agreed by many studies that NPs are delivered intracellularly preferably via clathrin- and caveolin-dependent pathways [55-57]. However, these conclusions are arguable, since caveolae is claimed to be the mechanism, only due to fact that NPs endocytosis can be reduced by cholesterol depletion. Or, alternatively, by assuming that NPs are co-localised with cholera toxin. Both statements are not convincing enough, because the agents pinching off cholesterol can interfere with other pathways, as well as cholera toxin is not taken up exclusively by caveolae. Worth to note, that endocytosis mechanism and efficiency are strongly dependent on cell type and origin. For example, the majority of cell types have equally charged surfaces, whilst epithelial cells are polarized and basolateral side has different properties than apical. Either poles of endothelial cells have different behaviour.

In summary, NPs are taken into the cells through clathrin-mediated mechanism, only if the particles form is optimal for invagination cluster. Once entered the cell, NPs are
trapped into a cargo vesicle called endosome. If endosome content can not be utilized by the cell, it'll be fused with peroxisome or lysosome to destroy it. Under acidic conditions in lysosomes, the molecular functional layer is getting pinched off and NPs start aggregation. The local massive release of toxic core ions may lead to oxidative stress induction and trigger cell death. The outcome of processes taking place in lysosome defines further cellular responses and cell fate. The described scheme is general approach, it varies depending on NP parameters and biological model.

1.2.2 Methods of investigation

Energy inhibition
Passive penetration of the molecules through the membrane is an energy-independent process, taking place either at low and normal physiological temperature. Endocytosis can be carried out only at normal physiological temperature (37°C). That's why first test on NPs internalising mechanism is uptake study at 4 and 37°C [58, 59].

Pharmacological inhibitors
To prove prevalence of one mechanism among others, pharmacological inhibitors are broadly used in study. However, the assumption that one drug can selectively block specific pathway is not fully feasible as often the same compound can cross-react with multiple receptors. Table 1.2 summarizes data about popular drugs used in the research, their cellular effects and drawbacks.

Table. 1.2 Common endocytosis inhibitors used in NPs uptake mechanism study (Adapted version from [60]).

<table>
<thead>
<tr>
<th>Toolbox of pharmacological inhibitors used to study endocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agent</td>
</tr>
<tr>
<td>-------</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>Action Description</th>
<th>Macropinocytosis</th>
<th>Additional Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amiloride (or its derivative EIPA or HOE-694)</td>
<td>Blocks macropinocytosis by lowering submembraneous pH (cytosolic pH close to the membrane) and preventing Rac1 and Cdc42 signalling</td>
<td>Macropinocytosis</td>
<td></td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>Inhibitor of Rho GTPase CME</td>
<td>Not efficient in all cell lines</td>
<td></td>
</tr>
<tr>
<td>Cytochalasin D</td>
<td>Inhibits actin polymerization and may thus lead to actin filament disassembly</td>
<td>Macropinocytosis and may affect several endocytic mechanisms</td>
<td>Not necessarily efficient in adherent cells, except for macropinocytosis</td>
</tr>
<tr>
<td>Latrunculin A</td>
<td>Sequesters actin monomers, blocks actin polymerization and may thus lead to actin filament disassembly</td>
<td>As for cytochalasin D</td>
<td>As for cytochalasin D</td>
</tr>
<tr>
<td>Jasplakinolide</td>
<td>Stabilizes actin and promotes actin assembly</td>
<td>Macropinocytosis</td>
<td></td>
</tr>
<tr>
<td>Dynasore</td>
<td>Inhibitor of dynamin function</td>
<td>Several mechanisms</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cholesterol depletion by extracting cholesterol</td>
<td>Macropinocytosis and both CME and CIE giving rise to small vesicles</td>
<td>Should be checked for possible leakage of K⁺ (more sensitive than protein leakage)</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------------------------------------------</td>
<td>------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Methyl-beta-cyclodextrin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filipin</td>
<td>Interacts with cholesterol A</td>
<td>Number of clathrin-independent and cholesterol-dependent mechanisms</td>
<td>Unstable and toxic</td>
</tr>
<tr>
<td>Nystatin</td>
<td>Interacts with cholesterol</td>
<td>As for filipin</td>
<td>Toxic</td>
</tr>
<tr>
<td>Lovostatin</td>
<td>Lowering of cholesterol content by inhibiting cholesterol synthesis</td>
<td>See pitfalls</td>
<td>Uncertain if agents inhibiting cholesterol synthesis are sufficient to inhibit endocytosis</td>
</tr>
<tr>
<td>Genistein</td>
<td>Inhibitor of several tyrosine kinases</td>
<td>Inhibits caveolae pinching. Used as a caveolae inhibitor, but not specific for this process</td>
<td>Affects several processes</td>
</tr>
<tr>
<td>Phosphoinositide 3-kinase inhibitors (wortmannin,LY94002)</td>
<td>Inhibit phosphatidylinositol 3-kinase</td>
<td>Macropinocytosis and compensatory RhoA mediated endocytosis</td>
<td></td>
</tr>
</tbody>
</table>
Cholesterol is an important mediator for caveolae and micropinocytosis. Overall, it is required for clathrin-dependent uptake too; thus cholesterol depletion will affect a number of pathways, and make it hard to say, which one is dominant. Some of the inhibitors can also be toxic and thereby affect cell viability.

**Expression of mutated proteins and use siRNA**

Alternatively, endocytosis can be disrupted by implementing mutations in the important proteins. As mutated proteins no longer have right structure, normal functioning will be corrupted, and the specific mechanism won't be available anymore. However, the side effects may reduce the effectiveness of chosen target. Small interfering RNA (siRNA) usually require few days (2-5) for cell treatment, and may impair other cellular functions and give non-relevant effects. Oxidative stress can be induced as the response to siRNA implementation. Overall, there is no guarantee that alternative pathways won't be elevated to cover knocked-down mechanism.

**1.2.3 Mechanism of QD uptake**

Current opinion suggests that QD endocytosis is strongly dependent on the particle’s surface chemistry. Typically QD size don't exceed 10 nm core diameter. It is therefore logical to assume that particles are trafficked to cells via clathrin-mediated route. However, the exact mechanism still remains unclear. According to leading opinion [61], the main condition for effective uptake is sufficient QD extracellular concentration. It's necessary to form vesicles with optimal size and shape that cellular membrane will wrap around and promote the intrusion of the QD into the cell. If the particle diameter deviates from optimal value, the uptake rate will be decreased. These statements were formulated on gold nanoparticles model [61]. Experimentally it has been discovered that the eligible size for quick and efficient uptake is 45-50 nm, which is at least 5 fold greater than QD core diameter [61].

Since QDs possess very small size, other factors also contribute to the difference in their uptake by cells. One of the crucial parameters influencing endocytosis is protein content in media. QDs bear different functional groups on their surface which in turn leads to protein sorption and corona formation [62-65]. It results in drastic increase in hydrodynamic size which can affect the process in certain ways, including mechanism switch and dynamics pattern change. More discussion is given in Chapter 4 [66, 67].
1.3 QD in vitro cytotoxicity

1.3.1 Factors influencing QD cytotoxicity

We should define first the meaning of cytotoxicity. As it comes from the name, it deals with something harmful for cells. The common definition of cytotoxicity is the degree of destructive action to the cell. In a first approach, it can be taken as ability of an agent to kill or, at least, reduce activity of target cells. More precisely, cytotoxicity can be tested by compound uptake and efflux, the interaction with extra- and intracellular receptors, the influence on growth and proliferation, metabolic activity, cellular compartments integrity, the mechanism of caused cell death (apoptosis/necrosis). A number of methods is applied to quantify cell activity. These include, but are not limited to: MTT, Alamar Blue, Trypan blue, Pico Green, LDH, Live/Dead, PI, Annexin V, TUNEL, Ki-67 etc (see List of abbreviations section for the full names of the methods).

Prior to any clinical application, the question concerning the safety of quantum dots must be fulfilled. The biggest concern relates to heavy metal content and high reactivity of nano-crystals. Unless various passivation techniques have been applied, there is no solidarity in reports investigating NPs’ toxicity. The contradiction in results arise from differences in biological models employed for the interpretation of studies. Other issues are diversity in experimental conditions, QDs characteristics, concentration and exposure time. The nanotoxicity is multi-parameter phenomenon, it depends on NPs size, shape, passivation, core/hydrodynamic size, surface chemistry, charge, presence of functional molecules (Fig. 1.3). Another issue making it difficult to interpret and comparison of obtained data is dose units (mg/ml, mol, ppm, number particles per cell); sometimes it's a time consuming and tedious task to correlate this crucial parameter across the studies.
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, NP type. There is a gross diversity in experiment design and conditions used in biocompatibility studies, from human cancerous cell lines, immune cells to primary mouse blastocyes and recently, primates [17, 68, 69]. At certain concentration/time point QDs induce cell death, which may follow 3 pathways: apoptosis, necrosis or autophagy. Classically, these mechanisms are alternate, however, in some papers apoptosis and necrosis take place in the same cell population [70, 71].

After trapping in primary vesicles (endosomes), nanoparticles are transported to cellular compartments. Usually NPs end up in lysosomes or peroxisomes with acidic conditions, as they can't be utilized by the cell. Introduction to a low pH and oxygen environment leads to NPs degradation and aggregation [41]. Since nanoparticles undergo deformation, the logical consequence is release toxic by-products. The most obvious reason of cell number decrease is leakage of Cd\(^{2+}\) from the QD core. It was very common in the first manufactured QDs which did not have protective shell, hence lots of surface atoms were available for ionizing in oxygen-containing media. Co-culture with any cells provoked massive release of toxic cadmium cations and resulted in quick cell death [41, 72]. Severe toxicity led to the demand of encapsulated QDs to enable their use in bioapplications. Interestingly, it was shown quantum dots have their own toxicity which is much higher than Cd\(^{2+}\) itself [72]. However, extra surface layers reduce this negative effect. It has been assumed that subsellular location of QDs is more important rather than ion cleavage. In other words, nanotoxicity is about where the leakage is happening, not \textit{how much ions have been released}. This was proved in later study by Chen et al in 2012 [19] who worked with core/shell/shell particles and found them almost neutral to cells. Based on that, they hypothesized that site-specific intracellular localisation of QDs leads to particles accumulation and massive release of Cd\(^{2+}\) in that area, which induce cell death (Fig. 1.4, from [19]).
Fig. 1.4 Scheme of CdTe QDs cytotoxicity. Engulfed nanoparticles allocated within cytoplasm in perinuclei area. Release of free cadmium ions is the main reason of observed toxicity; additionally, strict localization results in excessive toxicity in particular cellular compartments.

The capping layer can be built from cationic, anionic or zwitterionic compounds. Herein, listed types of functional groups have different impact on QD-cell interaction. For example, Tan et al [73] had generated and examined 6 different surface modifications. Their finding is aggressive behaviour of cationic and hydrophobic particles due to quicker internalizing by cells. Also they carried out control experiments using iron oxide NPs with similar surface ligands. Iron oxide NPs are recognised as non-toxic; however in this study they demonstrated the same trend in toxicity as QDs. This was attributed to ligand cytotoxicity. Another study done by Ryman-Rasmussen [74] questioned QD toxicity to human keratinocytes; in this case elevated cell death was registered for both amine and carboxylic acid coated particles, but not for PEGylated ones.

1.3.2 Mechanisms of cytotoxicity

ROS formation
Reactive oxygen species (ROS) is an abbreviation used to describe a range of molecules and radicals derived from molecular oxygen. ROS are produced by inflammatory cells and cause damage to DNA and proteins. This class of molecules is called free radicals (short-lived molecules with unpaired electron); most important for cell biology are
superoxide (O$_2^*$), nitroxy (NO$^*$), alkoxyl (RO$^*$), hydroxyl (*OH) and peroxyl (ROO$^*$) radicals and hydrogen peroxide ($\text{H}_2\text{O}_2$) molecule. The radicals have high reactivity, therefore they play an important role in cell defense against foreign bodies. Also ROS production triggers apoptosis. A curious fact, is that the mitochondria are at the same time the source and target of ROS. They oxidise cardiolipin-cytochrome c complex, what causes cytochrome c release, which, subsequently, activates cascade response from caspases-9,3,7, respectively. Fig. 1.5 shows the mechanism of ROS formation. The cells can be rescued from a lethal fate by antioxidants, such as catalase, N-acetyl cysteine (NAC), glutathione, alpha-tocopherol. Primary hydroperoxide radical (O$_2^*$) can be converted to less active hydrogen peroxide or neutral oxygen by superoxide dismutase enzyme (SOD). In turn, $\text{H}_2\text{O}_2$ can be either neutralised by defence mechanism (antioxidants) or converted into hypochloric acid (HOCl) by myeloperoxidase heme enzyme (MPD).

![Fig. 1.5 The mechanism of ROS formation and fate scenario. QD is effective electron donor, that can turn oxygen into hydroperoxide radical. After that, it can be transformed into toxic hydrogen peroxide. Antioxidants can block ROS promotion by converting $\text{H}_2\text{O}_2$ into water. Otherwise, $\text{H}_2\text{O}_2$ can converted into hypochloric acid or hydroxyl radical which resulted in cellular damage.](image)

Quantum dots are potent electron donors. They are able to induce molecular oxygen transformation to ROS and in turn, promote oxidative stress, cell damage or death [75]. Many studies reported QD cytotoxicity being mitochondria ROS mediated [42, 76, 77]. W-H. Chan [77] found that CdTe QDs cause IMR-32 human neuroblastoma cell death
via apoptosis by suppressing the expression of the survival signal and heat-shock proteins. Intriguingly, the exact apoptosis pathway is still under debates. While some groups provide evidences of classical caspase-dependent [77, 78] route, others see it as alternative caspase-independent process [42]. On the top of that, some studies found autophagy [79, 80] or necrosis as the prevalent mechanism.

**Apoptosis**

The first apoptosis induction by engineered iron oxide NPs was reported in 2004 by Berry et al [81]; earlier, in 1998, Xu et al [82] found that titanium oxide NPs caused cell death by necrosis in cancer cells. Apoptosis (programmed cell death) may be caused by a number of stimuli, internal and external. Apoptosis triggered by binding external ligands to the surface death receptor is called extrinsic. The intrinsic pathway is caused mainly by oxidative stress and is called mitochondrial route. Oxidative stress and ROS production are known to be apoptosis mediators. As it was mentioned above, mitochondria are both ROS target and source. Cytochrome c is an apoptosis mediator of key importance in the intrinsic (mitochondrial) apoptosis pathway. Cytochrome c is attached to the inner mitochondrial membrane via binding to cardiolipin. When cytochrome c gets detached from cardiolipin, as a response to oxidative stress, it is released through pores in the outer mitochondrial membrane formed by pro-apoptotic Bcl-2 family proteins and triggers caspase (cysteine proteases) cascades. Once released cytochrome c binds to a cytosolic protein Apaf-1, pro-caspase-9, dATP or ATP forming so called apoptosome complex. In this complex pro-caspase-9 undergoes processing and activation. Active caspase-9 cleaves and activates downstream caspases. Altogether, cytochrome c, caspase-9 and Apaf1 form a complex called apoptosome. The purpose of the executioner caspases-3,6,7 activity is to eliminate the cell. Interestingly, initiator caspase-8 has a dual regulation function; in necrosis it suppresses and in apoptosis it promotes the process. Fig. 1.6 shows machinery of both extrinsic and intrinsic apoptosis.
Fig. 1.6 Extrinsic and intrinsic pathways of apoptosis. The extrinsic apoptosis pathway is activated through the binding of a ligand to a death receptor, which in turn leads, with the help of the adapter proteins (FADD/ TRADD), to the recruitment, dimerization, and activation of caspase-8. Active caspase-8 then either initiates apoptosis directly by cleaving and thereby activating executioner caspase (-3, -6, -7), or activates the intrinsic apoptotic pathway through cleavage of BID to induce efficient cell death. The intrinsic or mitochondrial apoptosis pathway can be activated through various cellular stresses that lead to cytochrome c release from the mitochondria and the formation of the apoptosome, comprised of APAF1, cytochrome c, ATP, and caspase-9, resulting in the activation of caspase-9. Active caspase-9 then initiates apoptosis by cleaving and thereby activating executioner caspases. Taken from MacLlwan et al, 2013 [83].

The hallmarks of apoptosis are chromatin condensation, mitochondria swelling, membrane blebbing and integrity loss, nuclei deformation, cell membrane disruption. Figure 1.7 shows cytochrome c release mechanism followed by oxidative stress (from Orrenius&Zhivotovsky, 2005 [84]). Interestingly, ROS formation has opposite function depending on the process. For example, in eosinophils low concentrations of H$_2$O$_2$ are
the reason of apoptosis, high amounts do not affect cell cycle [85]. In a number of studies nitric oxide (NO*) has been shown as the apoptosis inducing agent for several cell types [86], while in other systems it blocked some apoptotic mediators [87]. It is also reported that ROS is necessary for cell surveillance. ROS role in apoptosis was discussed in more details [88].

In an earlier study HepG2 cell exposure to cadmium in a concentration as little as 2 uM, has resulted in apoptosis induction [89]. The process was found to be caspase dependent, with induction of capsase-3,8,9. Caspase-8 appeared to be an upstream regulator, as its activation was inhibited by zIETD-fmk specific inhibitor, but not by zVAD-fmk (broad spectrum caspase inhibitor). Release of proapoptotic agents Bid and Bax was hampered by treating the cells with NAC. In some experiments failure of zVAD-fmk to rescue the cells was related to a necrotic pathway, unless an elevation in caspase3/7 expression and change in mitochondria membrane potential were reported [90]. This conclusion is quite questionable, especially if common inhibitors

**Fig. 1.7** Cytochrome c release from mitochondria triggered by oxidative stress induction. Dysregulation in mitochondria membrane potential leads to cytochrome c leakage from complex with cardiolipin and caspases cascade initiation.
(glutathione, NAC) prevent cell death. Electron microscopy (in particular TEM) is a helpful method to resolve the question as it clearly shows some morphological alterations, which are very explicit for each death mechanism. Using same criteria – zVAD-fmk inhibitor activity towards caspase activity, Lovric et al, 2005 described the process taking place in MCF-7 cells (human breast cancer) as caspase-independent apoptosis. She also used unprotected QDs and has observed typical for apoptosis morphological changes – cell and mitochondria membrane dysfunction, nuclei damage, cytochrome c concentration increase in mitochondria, ROS formation; as in other studies, NAC was efficient reagent to prevent cellular damage.

When the same as used by Oh et al [89] hepatocyte cell line (HepG2) was treated with beta-cyclodextrin/glycirrhizic acid (beta-CD/GA) coated CdSe QDs, similar hallmarks of apoptosis were detected [91]. Nanoparticles selectively targeted hepatocytes among other tested cell lines (HeLa, Chang's liver, ECV-304); the uptake correlated with recorded cytotoxicity. Further investigation was taken for HepG2 cells and death mechanism was revealed. The comprehensive tests proved that mitochondrial dysfunction via ROS formation had its place, hence apoptosis is observed. Also cell cycle arrest is detected in G0/G1 phase. No information was provided about apoptosis mediators and how they related to ROS production.

Bare CdSe QDs were able to induce apoptosis in IMR-32 (human neuroblastoma) cells via caspase-3/9, but not caspase-8 activation and oxidative stress [77]. ROS were successfully neutralised by scavengers (alpha-tocopherol, NAC). The NPs activity is found to arrest survival pathway mediated Ras and Raf-1 molecules (it's resulted in reduction of ERK-1/2 protein activity) and down-regulation of heat shock protein 90 (HSP90). At the same time, ZnS-passivated particles did not affect cell activity.

In another malignant cell line, LNCaP (prostate cancer), the same mechanism of apoptosis (ROS mediated, caspase-dependent) has been triggered by CdS QDs [78].

Unlike the previous case, zVAD-fmk had effectively prevented caspase-3,8,9 downstream activity. Similarly, Bax/Bcl-2 ratio was increased, hence favoring apoptosis. DNA damage proteins were significantly upregulated (p53, HSP70), while NF-kB level (which is responsible for anti-oxidative cytokines activation) was dropped down. A free cadmium toxicity test was carried out and showed no effect on cells; the concentration of Cd^{2+} tested is the same as found to be leaked from nanoparticles. Thus it proves approach that cell death is rather related to nanotoxicity than cadmium toxicity. It renders Chen's assumption that QD subcellular localisation and massive
local increase of free Cd\(^{2+}\) concentration is the main source of nanotoxicity [19]. Earlier Su et al, 2010 [72] has pointed out that CdTe NPs toxicity is greater than free cadmium. More evidence in defense to the caspase-dependent pathway were provided by Yan et al, 2011 [92]. Human endothelial cells (HUVECs) were employed as a model representing response of blood vessels on QDs injection. The study was carried out in serum-containing (2% FBS) continuous culture (12-24 hours) conditions. The inhibitors zVAD-fmk and Ac-DEVD-cho have terminated caspase-3/9 activation, as well as NAC, and reduced QDs negative effect down to 40%. An interesting “threshold” effect is observed in ROS induction experiment: the highest introduced QD concentration (10 ug/ml, approximately 24 nM) found to down-regulate ROS amount, compared to control cultures, whilst lower doses (0.1 and 1 ug/ml) increased radicals content up to 135-138%. However, this effect has not been discussed. The authors also hypothesized that certain concentration may convert cell death mechanism from apoptosis to autophagy/necrosis.

Collectively, quantum dots are found to be powerful apoptosis inducers in various cell types (prevalently in cancerous cell lines). The hallmarks of apoptosis, either biochemical and morphological are listed below:

- Drop in mitochondria membrane potential (also occurring in necrosis)
- cytochrome c release
- pro-apoptotic Bax expression increase
- survival Bcl-2 down-regulation
- caspase cascade activation
- peripheral chromatin condensation. Looks like partial increase of chromatin density vs. complete bulk condensation in necrotic cells.
- DNA damage. So-called DNA laddering, unlike DNA smear which is characteristic for necrosis
- mitochondria swelling; also it happens in necrosis when all organelles are damaged.
- apoptosomes formation

These hallmarks are clearly observed after cell exposure to nanoparticles. In most cases, NAC and glutathione effectively rescued cells from the caused death. While not in all cases, pan-caspase inhibitor zVAD-fmk could prevent caspases activity. Researchers came to conclusion because (i) apoptosis is caspase-independent or (ii) cells follow
necrosis. In those cases no further investigation on apoptotic parameters were taken. It should be noted, that apoptosis is a complicated phenomenon, it can be induced by various signaling molecules and influenced by number of factors. The exact mechanism should be investigated in detail in each particular case. The presence of character signs should be confirmed, and the absence of alternative pathways should be proved too.

**Autophagy**

Autophagy is a mechanism of intracellular degradation of unnecessary or dysfunctional cellular compartments in lysosomes. The recycling of these components provides required energy for cell survival during starvation. All unused cellular components are wrapped into double-membrane vesicle called autophagosome. Next, autophagosome is fused with lysosome where further degradation takes its place. Engulfed nanoparticles either by passive or active transport are usually accumulated in lysosomes. The acidic environment of these compartments destroys foreign species which can't be utilised by cells. The biochemical hallmark of autophagy is the conversion of a light chain protein (LC3-I) to LC3-II by phosphatidylethanolamine (PE) conjugation. This results in autophagosome formation along with decrease in p62 expression (protein responsible for toxic cellular debris recognition). In low pH conditions functional molecules are eliminated from QD surface what inevitably leads to nanoparticles deterioration. It is still little known how aggregation affects cellular fate.

Since lysosomes have an important function in cell-QD interaction, they can moderate the outcome of nanoparticle uptake. In many cases NPs demonstrate high toxicity which resulted in cell death, autophagy becomes a probable scenario in the cellular response. The name of the process can be literally translated as “self-eating”. Autophagy describes the break-up of cellular entities (e.g. proteins, organelles) into components which can be used for further cell growth or homeostasis maintenance. It takes place in autolysosomes – compartments with double membrane, fusion of autophagosomes and lysosomes. The outline of pathway is showed in Fig. 1.8. This can be induced by a number of conditions, including oxidative stress, lack of growth factors, nutritional starvation, infection [93, 94]. In the previous section (see **Apoptosis**) mitochondria membrane permeabilisation has been pointed out as sign of the toxic effect caused by NPs. Similarly, here lysosome dysfunction is the result of membrane disruption caused
by trapped nanomaterial. In turn, lysosome membrane permeability (LMP) can induce ROS formation and mitochondria outer membrane decay. Depending on LMP breakdown degree, it can end up either by oxidative stress and apoptosis or cytoplasm acidification and necrosis. The mechanism and possible outcomes of LMP are illustrated in Fig. 1.8 and 1.9 [95]. Briefly, if lysosome membrane is not compromised much, it is followed by hydrolytic enzymes (e.g. cathepsins) release and consequently, mitochondria membrane disruption. More likely, it'll end up by triggering apoptosis. Otherwise, if LMP is massive, it can provoke ROS formation and involve mitochondria induced apoptosis or, if oxidative stress is abundant, the cell will proceed through necrotic pathway. However, autophagy is more pro-survival mechanism rather than cell death; observed lethal outcome of autophagy is artificially induced. The direct role of autophagy as “programmed cell death” is questionable.
Fig. 1.8 Autophagy mechanism. 1) During autophagy, a double layer membrane, the autophagosome, is formed that surrounds proteins and damaged organelles destined for degradation. 2) The autophagosome then merges with the lysosome, where hydrolytic enzymes in the lysosome dismantle the autophagosome contents. 3) The autophagy pathway is interconnected with the endocytosis pathways, with most endosomes eventually merging with the lysosome. The image is adapted from Stern et al, 2012 [95].
Figure 1.9 Mechanisms of autophagy and lysosomal dysfunction toxicity. The initiators of autophagy and lysosomal dysfunction toxicity, displayed in light blue text in the figure, include blockade of vesicle trafficking, lysosomal membrane permeabilization (LMP), and autophagy dysregulation. Nanoparticles could potentially cause autophagy dysfunction by overloading or directly damaging the lysosomal compartment, or altering the cell cytoskeleton, resulting in blockade of autophagosome-lysosome fusion. Nanoparticles could also directly affect lysosomal stability by inducing lysosomal oxidative stress, alkalization, osmotic swelling, or causing detergent-like disruption of the lysosomal membrane itself, resulting in LMP. Toxic effectors (lysosomal iron, cytosolic acidification, hydrolytic enzymes, reactive oxygen species, and the NLRP3 inflammasome) are displayed in dark blue. Conditions resulting from effector-mediated loss of homeostasis (oxidative stress, inflammation, ER stress, disrupted mitophagy, accumulation of ubiquitinated protein aggregates, and mitochondrial perturbation) are displayed in green. Finally, this loss of homeostasis can result in the cell death pathways necrosis, and apoptotic (type I) and autophagic (type II) cell death; displayed in red. From Stern et al, 2012 [95]
Quantum dots of different chemical composition are reported to induce autophagy in various cell types: porcine kidney cells [79], mesenchymal stem cells [80, 96], human glioblastoma [97], murine macrophages [98], GFP-LC3/HeLa [99], human cerebral endothelial cells [100], mouse renal adenocarcinoma [101]. As particles of similar size range were used and autophagy detected in all these cases, the nanotoxicity takes place rather than cytotoxicity caused by release of toxic agents.

Red-emitting (5.1 nm) CdSe and InGaP (3.7 nm) QDs significantly reduced porcine renal proximal cell (LLC-PK1) viability at concentration 10 nM after 24-48 hours exposure. The morphological changes characteristic for autophagy, such as double-membraned autophagosomes loaded with cell debris, have been found in TEM images. At the same time, no necrotic nor apoptotic ultrastructures were present [79]. Caspase-3 activity elevated at low concentrations was significantly shut down at toxic concentration values. Based on that, the authors assumed that cell death has not a caspase-dependent apoptotic nature. Then, no correlation between oxidative stress and metal-induced toxicity has been found. Similarly, cerium oxide (CeO$_2$) NPs induced both caspase-independent apoptosis and autophagy in primary human monocytes [102]. The autophagy was proved as a cell death pathway not only co-existing, but cross-talking to apoptosis. Autophagy inhibition is resulted in increased cell survival rate, whereas p53 silencing (which regulates both processes) amplified autophagy and did not affect apoptosis. Controversially, Luo et al, 2013 [101] showed on mouse renal adenocarcinoma cells (RAG) that NAC and 3-MA inhibited ROS formation and autophagy, hence increased number of apoptotic cells. They've hypothesized that moderate oxidative stress is essential for autophagosome formation, whereas massive ROS production drives cells to apoptosis. It contradicts to Stern et al, 2012 [95] who postulated that autophagosome/lysosome dysfunction is underlaying factor of oxydative stress and depends on LMP degree.

The variation in nanoparticle size from 3.7 to 60 nm did not affect the cell response [79, 97, 102]. Thus, we can assume in case of NPs we're dealing with nanotoxicity rather than classical cytotoxicity. One of the main contributing factor, driving cells either to apoptosis or necrosis is the particles uptake rate. Indeed, if observed effects are not caused by leakage from the NPs core, it is known that the accumulation of trapped ubiquinated protein-NP complexes is the reason of autophagosome dysfunction; then, after autophagosome-lysosome fusion, the degree of its rupture (and further cell response and fate) depends on protein-NP content. In fact, cells do not ingest NPs
equally, thus it's the reason of realising of 2 different death mechanisms at the same time in the same cell population.

Bigger species, like graphene oxide sheets (350 nm – 2.4 um effective hydrodynamic radius, 1-2 nm thickness), do induce autophagy in murine macrophages and immune responses similar to bacterial intrusion through TLR4 and TLR9 [98]. However, cell number did not decrease even after 24 hours of exposure to the material, mainly due to species dimensions not favourable for uptake.

Necrosis
While apoptosis is a programmed tightly controlled on all stages process, necrosis is violent cell death. It affects a group of contiguous cells and caused by external agents followed by severe inflammation. The hallmarks of necrosis are disintegrated cell membrane, random DNA damage, organelles swelling and perforation, cell lysis and phagocytosis by macrophages.

Necrosis is a passive process required direct intervention into cell cycle, like infection or toxic chemicals. For example, it can be the dissolution of exposed agents. Large calcium phosphate (CaP) NPs (300 nm diameter) were found to cause necrosis in HepG2 cells via lysosome rupture and release of hydrolytic enzymes, such as cathepsin B and D [103]. However, particles did not disturb lysosome integrity in hypertonic solution (145-325 mM NaCl). The lysosome disfunction was induced by an osmotic pressure disbalance. The excess of free cytosolic Ca²⁺ originates from CaP NPs dissolution. Thus, hypertonic conditions restore osmosis and keep intracellular calcium low.

In the case of shielded QDs, the necrotic pathway is more related to functional layer toxicity factor than to core itself. The role of ligands length/charge and QD diameter on cell viability and proliferation was investigated by Nagy et al, 2012 [104] and summarised in Table 1.3. Briefly, the surface charge is the most influencing parameter for QD biocompatibility, carrying positive charge particles are extremely harsh on the cells. After the charge, the length of the ligands significantly shifts number of viable cells. In my opinion, “ligand length” correlates with hydrodynamic radius, as it adds the thickness to QD core.
Table 1.3 QDs cytotoxicity is dependent on particles size, surface charge and ligand length

<table>
<thead>
<tr>
<th></th>
<th>long ligands</th>
<th></th>
<th>short ligands</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MUA-QDs</td>
<td>AUT-QDs</td>
<td>MPA-QDs</td>
<td>CYST-QDs</td>
</tr>
<tr>
<td></td>
<td>3 nm</td>
<td>5 nm</td>
<td>10 nm</td>
<td>3 nm</td>
</tr>
<tr>
<td>cell response</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>necrosis</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td>++</td>
</tr>
<tr>
<td>apoptosis</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>proliferation</td>
<td>---</td>
<td>---</td>
<td>0</td>
<td>---</td>
</tr>
<tr>
<td>ROS</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

MUA - mercaptoundecanoic acid
MPA - mercaptopropanoic acid
AUT - amino undecanethiol
CYST – cysteamine

The number of + corresponds to the number of concentrations that resulted a significant increase in response. The number of - corresponds to the number of concentrations that resulted in a significant decrease in response. The fields marked with 0 indicate that none of the concentrations tested resulted in a significant increase or decrease in response. Taken from Nagy et al, 2012 [104].

The interplay of pro-survival and lethal mechanism realised in cells in response to intruders. Necrosis is a secondary mortal route which can be triggered as last instance of QDs exposure. In a number of studies, a necrotic subset of cells is found to co-exist with apoptotic. Apoptosis and necrosis are not fully independent, but cross-connected processes. For example, caspase-8 serves as initiator in apoptosis and suppressor in necrosis. Or, NAC, can rescue cells from both mechanisms co-existing at the same cell population, as observed in HEK293 cells (human embryonic kidney) exposed to yttrium oxide NPs [105]. Apparently, it happens due to the ROS scavenge effect triggered by anti-oxidants. The phenomenon of such differentiation remains unclear; perhaps the reason lies in NP uptake difference by individual cells which results in non-equal ROS.
production hence different death mechanism. One could suggest that the processes take place consequently. For example, a cell goes first through apoptotic cycle, when it has reached late stage it can be cleaved by necrosis. However, it is not feasible assumption, as oxidative stress is trigger for both, and same caspase-8 can either promote apoptosis and suppress necrosis. Thus the cell is facing a choice on early stage, as both routes can't be triggered by the same mediator. To consider a caspase-independent apoptosis (what is quite questionable, as each caspase inactivity requires a proof), then the above scheme can be executed, but it suggests less controllable scenario. A useful experiment could be done to unveil the reason – if NP treated cells were PI/Annexin V stained and sorted on FACS and then ROS level measured for four categories of cell subsets: live, early apoptotic, late apoptotic and necrotic. In this case, the ROS expression would give answer wether oxidative stress defines the appropriate death mechanism or not.

Another recent study on a necrotic pathway used murine macrophage model (RAW264.7 and J774A.1 cell lines). It has revealed TLR4 mediated route trigger is caused by the treatment with carbon oxide derived engineered nanomaterials [106]. The involvement of pro-inflammatory machinery is a typical response of immune cells to intruders; TLR4 activation is associated with a defense against LPS. When TLR signaling was blocked, the number of dead cells has significantly dropped. It should be noticed, that graphene oxide nano-scaled derivatives predominantly cause necrosis, whereas the apoptotic subset was very small (<5%). Taking in consideration cases above, the same result (apoptosis/necrosis induction) varies depending on cell type.

1.3.4 Cytotoxicity of QD bio-conjugates

As it was mentioned above, QD is prominent candidate for molecular fluorophores alternate (see 1.1.2 Optical properties section for detailed comparison). Quantum Dots have been used as fluorescence reporter in molecular beacons [107-109], antibodies [110-113], anti-cancer drugs [11, 114-117], proteins [118, 119], peptides [120], biomolecules [121]. On another hand, toxic effects of QDs have been examined in numerous studies. However, only few works have considered and reported hybrid probes cytotoxicity.

All described tests can be divided into two groups: acute and chronic exposure. Acute exposure is usually less than 4 hours [117, 121-123], whereas chronic introduction is from 24 to 96 hours [124-127]. In most cases, cell viability is greater than 80% [128, 129]. The majority of conjugates are designed for bio-imaging and live cell targeting,
thus low toxicity is required [130, 131]. However, some constructs are aimed to target and destroy cancer cells, thus QDs were engrafted with peptides and drugs [114, 124]. It should be noted, that comparison is quite difficult due to various units used to express conjugates concentration – ng/ml, ug/ml, nM, percents. Interestingly, there’re many examples when conjugates have better biocompatibility compared to QDs. This is due to obvious fact, that functional molecules (such as hyaluronic acid, coenzyme Q, thymidine kinase, lactose) provide extra layer of capping and increase hydrodynamic size [121, 126, 127, 130]. Also attachment of specific molecules requires receptor recognition, that enables selective targeting according to markers affinity to the cells [122, 124].

1.4 Immune responses caused by nanoparticles

Unique properties of nanoparticles have been discovered in the late eighties of the last century, but the peak of their vast production and application started in mid nineties. Their exceptional characteristics are as a result of the combination of complicated surface chemistry, small size, chemical composition, shape, affinity to water, organic solvents and the behavior in colloidal systems. In last two decades nanomaterials have been widely used in catalysis, solar cells, cosmetics, sunscreens, paints, medical and electronic devices. However the safety question of manufactured NPs remains open. Due to fact of humans introduction to them mainly through the airway, contact with skin and mucosal surfaces, nanotoxicology has become the major concern.

Being the first gates in innate immune response chain, macrophages play important role in inflammation initiating, development and fate. Broad repertoire of subsets provides steady defense against invading foreign entities. The primary reaction of macrophages is the trapping of entering species recognized as non-self bodies; further in depend on stimuli nature, number of immune cascades can be triggered, likewise up-regulated expression of pro-inflammatory interleukins (IL-1, IL-6), tumor necrosis factor alpha (TNF-α), reactive oxygen species, cyclooxygenases (COX). Beyond that, some nano and fine particles are able to quench aforementioned responses which leads eventually to failure of macrophage functions.
1.4.1 Carbon black (CB) nanoparticles

Carbon black consists of elemental carbon in the form of combustion-derived colloid or a suspension produced by partial thermal decomposition particles, either in gaseous or liquid phase. Physically it is an extremely fluffy powder with a large surface area, consisting of aggregates of three particle fractions: nano-scaled, ultrafine and fine. It has been reported to be an industrial air pollutant causing allergic airway diseases \[132, 133\] and as potential cancerogen in mice and human models. In the meantime it should be taken into consideration that mainly high toxicity of CB is caused by various aromatic by-product compounds attached to a particles’ surface. The in vitro study showed the induction of ROS generation, NF-kB activation and enhancing of cyclooxygenase-2 (COX-2), TNF-\(\alpha\) and IL-6 production \[134\]. The model used in the experiments is murine monocyte-macrophage cell line RAW264.7.

Nano-sized CB particles (CBNPs) appear either as single or multi wall tubes, with diameter ranged from 20 to 200 nm. Applied along with OVA as allergy booster, CBNPs were shown to trigger an inflammation in lungs in vivo. It was accompanied by high release of IgE, which led to an increase in number of eosinophils and induction of Th2 cytokine production (IL-5, IL-10); this took place after 21 days of exposure \[133\]. In earlier study mice were exposed to multi-walled NPs by inhalation during 7 and 14 days. A significant increase in IL-10 expression was indicated only after 14 days of treatment where no signs of sufficient inflammation were detected, but IL-6 level was not elevated. High rate of uptake by the lung macrophages and escalated pathological alterations in histology have been shown \[135\].

CBNPs can affect lung macrophages either directly or indirectly. Type II rat alveolar epithelial cells (L-2) are known by their ability to mediate macrophage migration through the expression of chemoattractant molecules like as MCP-1, IL-8, TNF-\(\alpha\) \[136-138\]. Due to the high surface-to-volume ratio, CBNPs and TiO\(_2\) NPs induced lactate dehydrogenase (LDH) release in L-2 cells line. However, only exposure to carbon particles resulted in chemotaxins up-regulation and macrophages recruitment to the stimulated site \[139\]. TiO\(_2\) particles, as well as fine CB particles were proved as inert.

Particle shape also contribute to the cell-nanoparticle interaction. CB aggregates consisting of straight tubes tend to avoid phagocytosis and stimulate oxidative stress. This may lead to chronic inflammation; in contrast, nanofibers can not promote release of peroxide nor TNF-\(\alpha\). After all it does not mean that tangled structures are fully
harmless. Short-term in vitro experiments revealed the ability of tangled CBNPs to inhibit activation and phagocytosis of E. coli by THP-1 cells [140]. Overall carbon black species may cause pathological alterations in lung tissue to be one of reasons of cancer, but it can develop without acute inflammation. The residue of impurities on the particles surface, size, aggregation, primary type of graphene structure and shape of aggregates contribute to complexity and variability of interaction with macrophages.

1.4.2 Silica nanoparticles

Fluorescent-tagged silica NPs have been proposed as a beneficial tool for bio-imaging, cells labeling [141-143], drug carriers for the controlled release and bio-sensing [144]. This interest is based on the assumption that they don't contain any toxic ions in the core (no Cd, Te, Se, As). Silica NPs are potentially inert and don't require any surface passivation. Nevertheless it should be admitted that the lack of negative influence on cells viability does not guarantee absence of immune reactions which required longer time to be triggered rather than necrosis.

The main trend in Si NPs uptake and inflammatory potential increase with decrease in particle size and concentration [145, 146]. Indeed, there is nothing unusual in the fact that particles with greater surface and bigger number of reactive atoms have high reactivity. It leads to leak of aggressive radicals into cell cytoplasm and trigger inflammation as defense mechanism. Apparently that smaller particles are ingested quick and in high rate. Further was demonstrated the necessity in capping Si NPs by neutral or negatively charged (amino-terminated) surface layer to prevent their penetration in nuclei and reduce cells damage [146, 147].

Interestingly enough, studies of immune reactions in macrophages consider only over-expression of cytokines (IL-1, IL-6, TNF-α) and ROS (NO, peroxidase) but they don't give any comments when these parameters are notably lower than in untreated cells [145, 146, 148]. This is the most intriguing part – certain exposure time and particle characteristics (composition, surface chemistry, size, concentration) suppress the inflammation whilst it is supposed to be enhanced. Why does it happen? Does it mean that macrophages lost their protective ability? How does it affect further fate of particles and other immune cascades? All these questions still remain open.
1.4.3 TiO$_2$ nanoparticles

The risk of casual exposure to CB, carbon nanotubes or nanowires, CdTe, CdSe, Si and other toxic NPs is very low. However, every day we consume TiO$_2$ containing goods. This is widely used type of nanoparticles. The broad implementation of this material is started from white pigment for paints. It is a good alternate to antimony- or lead-containing compounds used before for a long time before they appeared to be toxic. By now TiO$_2$ is a common filler used in food and cosmetic industry. Thus the questions of safety, stability under physiological conditions and final fate of TiO$_2$ NPs in biological systems are under intensive research.

An in vivo study for single intratracheal instillation showed drastic enhancement of multiple inflammation parameters, such as IL-1, 4, 5, 6, 10, 12, TNF-alpha and IFN-gamma [149]. The responses were acquired at 1, 7 and 14 days post-instillation. Acute production of inflammatory cytokines in bronchalveolar lavage (BAL) fluid and blood was observed at day 1 in 20 mg/kg treated group. The extremely high expression level of a number of cytokines, such as IL-6, IL-10, IFN-$\gamma$, IL-2 and IL-4. These cytokines stimulate T-helper type 1 (Th1) cell proliferation, which triggers anti-bacterial defence mechanism. It was observed up-regulated expression of genes which are responsible for inflammation and induction of chemotaxis of immune cells. Also has been detected the formation of granuloma in the lungs, intensive secretion of macrophage inflammatory protein-2 (MIP-2) and monocyte chemoattractant protein-1 (MCP-1) at day 1 and 14, respectively. This is the clear evidence that acute inflammation is gone to chronic form. The sufficient increase in IgE level in BAL fluid and blood is sufficient evidence of allergy sensitization. Earlier study explored the effects of inhaled NPs. Was observed up-regulation in some chemokines expression (CXCL1, CXCL5 and CCL3), which resulted in alveolar epithelial cells damage and apoptosis. Emphysema injury accompanied by activated macrophages infiltration was also found [150].

Small (2-5 nm) titanium dioxide NPs were administrated through inhalation [151]. Mice were exposed acutely (once for 4 hours) and chronically (4 hours/day during 10 days) to TiO$_2$ containing aerosol. Acutely treated mice did not show any significant inflammation in the lungs and BAL fluid. The animals, which were chronically exposed to nanoparticles for 1 and 2 weeks, had a slight increase in IFN-$\gamma$, IL-6 and IL-1$\beta$ concentration in BAL fluid. After 3 weeks of exposure mice recovered and the
chemokines expression has returned to normal values.

1.4.4 Silver and Gold nanoparticles (Ag and Au NPs)

Gold nanoparticles have been widely investigated due to their strong absorption and scattering properties, high biocompatibility and convenient surface chemistry. The surface of gold NPs can be decorated with proteins, antibodies, drugs, nucleotides, organic dyes and surface markers. It is possible because of high affinity of Au NPs to amine and thiol groups. These ligands form clusters, which in turn compose a protective shell around the particle. Lack of toxic ions, excellent size-tunable optical properties and ease in functionalizing made Au NPs an effective tool in a number of bio-medical applications. This includes cancer therapy [152], drug cargos and controllable release systems, carriers for precise bio-targeting, cells tracking and bio-sensing [153]. Antibacterial and antifungal properties of silver NPs make them a convenient biomaterial for wound dressing, contraceptive devices, surgical instruments and masks, bone cement and prostheses. Silver nanoparticles are also applied as coating material for household and industrial appliances. Bactericidal activity of Ag NPs strongly depends on their shape rather than size, as it was shown in study with E. Coli [154].

Similar to silica, spherically shaped Ag and Au nanoparticles cause up-regulation of pro-inflammatory cytokines (IL-1, IL-6, TNF-α) at short-term treatment (3-6 h) and arrest their production at long-term exposure (24 hours and more). The difference between gold and silver NPs is in ability to induce immune responses. Gold NPs elevated the expression level of pro-inflammatory genes in size-dependent manner in murine alveolar macrophages J774 A1 [148]. Human monocyte cell line THP-1 demonstrated similar response; treatment with high concentration of the particles resulted in over-expression of IL-6, IL-10 and TNF-α.

The antimicrobial activity of Ag NPs has been compared to commercial antibiotics. This is tested on a number of common bacterial strains (E. coli, P. aeruginosa, E. faecalis, S. aureus, S. maltophilia and C. jejuni). The results revealed that Ag NPs are more effective than any tested antibiotics [155]. RAW264.7 murine macrophage cell line was used to examine immune reactions in response to the different NPs [134]. It was found that most types of NPs increased intracellular ROS content, but in the same way (2.2-3.8 ± 1 folds higher than in control
cultures). Only gold particles have down-regulated oxidative stress. The treatment with lipopolysaccharide (LPS) caused more strong ROS production (5.8±1.25 folds). Silver and aluminium NPs activated NF-kB signaling pathway; this mechanism is responsible for cells survival. Also they increases expression of cyclooxygenase-2 (COX-2), IL-6 and TNF-α. These inflammatory cytokines were up-regulated only after long-term co-incubation with the cells (48 hours). At the same time, gold NPs did not affect profile of these cytokines.

Unlike a murine model, rat alveolar macrophages did not show any sensitivity towards Ag NPs. The particle size ranged from 15 to 55 nm [156]. Surprisingly IL-6 expression level has not changed at acquired time points (6 and 48 hours).

1.4.5 Iron-containing nanoparticles (Fe NPs)

Magnetic resonance imaging (MRI) is convenient technique for non-invasive soft tissue imaging without usage of radioactive isotopes or X-rays. Nevertheless MRI suffers from low sensitivity and hence gives poor resolution results for minor tissue alterations. Paramagnetic particles seem to increase contrast and therefore the sensitivity. The rare-earth elements family (are also called lanthanides, the row in the Periodic Table La-Yb including scandium and ytterbium) are known for excellent magnetic properties of their derivatives. That’s why gadolinium (Gd) compounds were one of the first explored as contrast agents. The efficiency of Gd-containing complexes still remains relatively low, have short circulation time and may leak toxic gadolinium ions. Thus a search of more effective alternates is of high priority. Iron oxide materials are eligible candidates due to the set of paramagnetic characteristics combined with good stability in biological systems [157, 158].

The ex vivo model was used in the study. Primary peritoneal macrophages were harvested from mice and exposed to iron-containing particles. The iron oxide NPs induced pro-inflammatory response in a dose-dependent manner [159]. Cell viability was not affected, only relatively high concentrations (>100 ug/ml) promoted cytokines (TNF-α, IL-1β) and NO secretion, but less significant than LPS. Chemotaxis was reduced by the arrested expression of CX3CL1 chemokine. The earlier research with the same mice strand (C57BL/6) did not reveal any inflammatory effect [160]. The murine macrophages demonstrated increase in IL-10, while no deviation has been observed in
the rat model. Human Monocyte-Macrophages (HMMs) were examined in vitro for their susceptibility to Fe$_2$O$_3$ NPs [161]. Cells remain indifferent to stimulation with iron NPs, none of examined cytokines (IL-12, IL-6, IL-1β, TNF-α) was elicited. In addition, loading cells with NPs did not affect their ability to be activated by LPS. As in the case with the murine model, chemotaxis in HMMs was arrested too. The oxidative stress wasn’t induced.

1.4.6 Cerium oxide nanoparticles

Most of the above discussed particles were considered as potential reasons of the inflammation caused by foreign bodies. In contrast, cerium oxide nanoparticles have been proposed as potential NO scavengers. This feature could be implicated in treatment of chronic inflammation. Cerium oxide particles have oxygen defects at the surface. This kind of the surface defects is typical for this compound because of irregularities in lattice. Thus it creates traps for oxygen-derivated radicals. The ability of nanoceria to decrease NO production in the J774 A.1 murine macrophage cells line followed by stimulation with LPS and IFN-γ [162]. In addition, was confirmed reduction in iNOS expression - the gene is known as being responsible to trigger NO production. An in vivo test did not demonstrate any significant difference between control group and mice received high dose intravenously. The traces of granulated nanoceria were found in blood vessels tissues, liver hepatocytes and renal tubular epithelia cells in high-dose long-term exposed mice. All others main organs – lungs, brain, pancreas, liver, kidneys, spleen – did not show any evidences of pathological alterations.

1.4.7 Conclusion

Discovery of unique possibilities brought by nano-scaled particles of well-known bulk materials (such as silica, carbon, titanium dioxide and etc) caused a vigorous boost in technology and changed our views on existing matters. Engineered nanoparticles made an essential impact in the majority of biological and medical applications, but the issue
arose with potential risks of their exposure to humans. Due to the complex nature and sophisticated organization of immunity, various responses to NPs might be revealed on different levels of the immune system over long period of post-treatment times. Macrophages are the first line in an innate immune defense; their inherent function is the physical isolation of foreign bodies by ingestion and destruction. If for some reason the ingested species can not be neutralized by macrophages, inflammation has to be initiated. The signaling cascades of presence of potentially dangerous entities are propagated and activate next level of protective mechanism. The nanoparticles can not be destroyed easily by cells due to their engineered nature. That’s why they cause promotion of inflammatory reactions and activate macrophages. But at some point NPs start to quench pro-inflammatory cytokines and surface markers expression. Currently, there is no clear answer regarding the nature of this phenomenon. This section has sought to show general trends in interaction between various types of macrophage cells and nanoparticles.

1.5 Ternary Quantum Dots

1.5.1 Benefits and prospective
To facilitate deep tissue penetration required for effective in vivo bio-imaging, QDs should have an emission peak in near infra-red (NIR) spectrum. One of the most striking opportunities can be achieved by implementing NIR QDs is optically guided surgery. The clinical translation of widely investigated QD imaging technique is hampered because of toxic components of the nanoparticles (Cd, Te, Se, Pb, Hg). The convenient Cd-based QDs are also limited in the quantum yield and fluorescence intensity in NIR region. Thus alternative materials should be explored to overcome this issue.

The choice has fallen at I-III-VI$_2$, V-VI-VII or III-V semiconductor classes. The principal difference lies in chemical bonds of the core [163]. Unlike II-VI crystals which have ionic bonds, III-V compounds are described by robust covalent bonds, what makes the NPs less toxic. Ternary semiconductors have band gap width similar to binary II-VI (1.45-1.8 eV), their luminescence ranges from visible to the near infra-red (650-900 nm) spectrum [164-167]. Small lattice mismatch value (<2%) allows epitaxial growth of thick ZnS shell which significantly strengthens particles stability under
physiological media conditions [164]. However, similar to II-VI NPs at the early stage of research, synthesis and stabilization of ternary QDs requires intensive improvement. To date, hot injection in organic solvents remains as the most popular synthetic route.

1.5.2 Antimony sulfo-iodide

Antimony sulfo-iodide (SbSI) is widely studied as it has many unique properties including semiconducting, ferroelectric [168-171], photoconductivity [169, 170], piezoelectric, pyroelectric and pyro-optic [169]. Its ferroelectric properties change when it is optically excited as its adsorption edge is in the near infra-red region, so SbSI is said to be photoferroelectric. SbSI has the highest Curie temperature ($T_C$) of any of the V-VI-VII class of ternary materials is approximately 21°C [168, 170, 172]; the highest dielectric constant along the polar axis of $5\times10^4$ and also the highest known refractive index (n) of any material of 4.5 along the c-axis [170, 173]. This refractive index adds to the importance of SbSI in the development of electro-optic devices, although this is dependent on large defect-free crystals becoming readily available. SbSI has a band gap of 2.12 eV, which shows abnormally high temperature coefficients [171]. It is also a p-type semiconductor [174]. These properties of SbSI make this material very promising for applications in displays and solar cells. SbSI is also a promising candidate for use as infrared detectors in thermal imaging [168]. The small size particles of SbSI are can also find important applications for future developments of nanoscale electronic and optical devices [175].

Antimony trisulfide (Sb$_2$S$_3$) is a very important intermediate as its formation is a key step in the preparation of SbSI. Sb$_2$S$_3$ possess good photovoltaic properties and has high thermal electric power, as a result it has been applied for use in television cameras, electronic and optoelectronic devices and also in infrared (IR) spectroscopy [176]. Sb$_2$S$_3$ lattice is orthorhombic, there are four molecules per unit cell, it has a crystal structure Pnam [177]. SbSI is also orthorhombic but differs in an axis of symmetry and has a different lattice type (Pnma).

The era of fluorescent nanoparticles, so-called Quantum Dots (QDs) has started from exploration of size-tunable luminescent properties of II-VI class semiconductors on nanoscale level. QDs have gained lots of attention as prominent bio-imaging agents. The gross advantage of QDs versus organic dye is the set of unique properties [38]. They demonstrate bright size-dependent photoluminescence, broad absorption, large
Stokes shift, high quantum yield and long life-time. Numerous studies have revealed large potential of QD in vitro and in vivo applications, however, their use in humans is hampered due to presence of toxic components (Cd, Se, Te) in the core. Moreover, QD fluorescence is limited in near infra-red region eligible for non-invasive clinical bio-imaging. Recently, ternary semiconductors have been pronounced as non-toxic candidates for NIR emitting quantum dots. A few papers reported successful synthesis and in vivo application of water-soluble NIR nanoparticles made of CuInS$_2$ [164, 178-180].

Various techniques have been developed to produce and texture SbSI in its different modifications. Flash and beam evaporation methods were first reported for thin film manufacturing [181-183]. Hydrothermal synthesis, sonication in ethanol, vapor transport reaction, refluxing were approached by number of groups for SbSI and its derivatives [169-171, 175, 176, 184-186]. Unless all gained features all mentioned methods suffer either from long reaction time, high temperature/pressure, low product yield, small synthesis scale. Simple and elegant water-based synthesis route has been described by I. Rybina in 1998 [187]. It allows to produce pure crystal SbSI from easy available precursors in large scale at room temperature. The only downside of this technique is the poor control of reaction kinetic and thus crystal size/shape.

The main goal of our work was to develop and optimise the synthesis of SbSI micro- and nanocrystals, investigate their optical properties and perform in vitro biological testing. In current study we also explored influence of physical and chemical parameters (temperature, solvent, ionic strength, pH) as well as presence of surfactants on synthesis kinetic, crystal formation and particles dimensions. The reaction is found to be extremely sensitive to pH and solution ionic strength. Treatment with ultrasound helps to improve final product purity and crystallinity. Crystal shape has been drastically changed upon adding surfactants. The cytotoxicity of as-prepared material also has been tested.

### 1.6 Methods of QD quantification

Upon exposure to biological media conditions, NPs undergo number of consecutive alterations, mainly resulting in particles degradation. In many cases, NPs before and after adding into bio-systems are not the same species. That's why NP quantification in biological models is a very challenging task. The importance of correct intracellular QD content can not be highlighted enough, as their biomedical applications strictly demand
easy, fast and accurate data interpretation.

1.6.1 Light microscopy
All quantification methods can be divided into two groups: sample preserving and sample digesting. Various microscopy techniques (light, fluorescent, confocal, electron) are aimed to preserve the cell integrity and acquire NPs within the cell boundaries. It also allows precise detection of NPs co-localisation with the specific organelles. However, fluorescent microscopy is not able to distinguish whether particles are adhered to the surface or inside the cell. A second downside is the lack of accuracy, as it based relative signal intensity, hence doesn't specify how many particles per cell. The discovery of super-resolution microscopy has decreased the detection limit to as little as 50 nm. Stimulated emission depletion (STED), stochastic optical reconstruction, photoactivated localisation microscopy are the new approaches enabled visualisation of individual nanoparticles of corresponding size [188-190]. For example, STED has been used to distinguish and quantify 25 nm and 85 nm silica nanoparticles internalized by A549 epithelial cell line [191]. Fig. 1.10 shows confocal versus STED images of 40 nm beads (from Willig et al, 2006 [190]).

![Confocal versus STED images](image)

**Fig. 1.10** The advantage of STED over confocal microscopy. (a) confocal microscopy image (b) STED image of 40 nm bare beads. Scale bar is 500 nm.

1.6.2 Flow cytometry
Flow cytometry doesn't require cell lysis in a prior assay; it also suggest to sort the cells according to their physical parameters (size, granulation). Similarly to light microscopy, it only measures relative fluorescence intensity per cell. Calibration of the signal is the
most significant issue of the method. Flow cytometry is convenient approach for semi-quantitative nanoparticles assay and can be used along with biological markers. Thus, it can be applied to simultaneously to measure the uptake along with cellular responses. Moreover, fluorescence activated cell sorting can be used to select and sort cells containing QDs for further culture and experiments.

1.6.3 Electron microscopy
Transmission Electron Microscopy provides exceptional resolution in visualising nanoparticles inside the cells. The biggest disadvantage of the method is time consuming sample preparation which require a large number of cells. Also the process involves highly toxic osmium tetraoxide as contrast increasing agent. In most cases cell sectioning is not consistent – it often does not go through the middle, thus some results might be false and contain no nanoparticles. It can be overcome by using automated systems which can consistently cut and image each section to make 3D reconstruction of the entire cell [192]. Only thin sections of biological samples (less than 300 nm thickness) can be examined. Imaging of thicker sections require higher beam voltage what results in fragile samples melting.

1.6.4 Mass spectrometry
The family of methods based on mass spectroscopy employs cell digestion prior to quantifying QDs. These techniques have a very low elemental detection limit which results in an accurate mass, and, consequently, number of QDs, evaluation. At the same time, it's not possible to say how many QDs are in the individual cell, only estimated average value is obtained.
To date, inductively coupled plasma (ICP) mass spectorometry (ICP-MS) and atomic emission microscopy (ICP-AES) are the absolute standard for precise nanoparticle quantification inside the cells [193, 194]. This technique (ICP) is based on high temperature electromagnetically induced argon plasma (6000-10000K), which is sufficient to break down most bonds. ICP-AES recognises and resolves elemental content by its electromagnetic emission spectra indicated from atom excitation by plasma. In ICP-MS samples are atomized and ionized by induced argon plasma and mass-to-charge ratio are analysed by mass spectrometer. Both techniques require cells
in single cell suspension, with live cells pre-sorted if desired. However, in biomedical application, intracellular QD content should be quantified as number of nanoparticles per cell. Desirably it should reflect population behavior upon exposure to nanomaterial. Both techniques are capable to provide information of general QD amount in the probe, as it requires sample full dissolution. Yet it can't analyse QDs in individual cells.

1.6.5 Mass cytometry

Mass cytometry is a novel method combining the advantages of flow cytometry and inductively plasma generated mass spectrometry (ICP-MS). Instead of convenient molecular fluorochromes, it explores how stable isotopes can be used to tag the cells and takes readings with mass spectrometer. Isotopes are easy to resolve from each other as they have unique masses what can be reflected in ICP-MS spectra (Fig. 1.11 from Tanner et al, 2013 [195]). Each isotope has its own unique spectrum. There is no overlap during the measurements. Unlike convenient molecular dyes, which suffer from fluorescence spillover and require careful compensation; it significantly restricts number of colors can be used in assay.

![Mass spectrum of 30 enriched stable isotopes of the lanthanides, recorded for solution analysis at concentrations of approximately 20 ng/L (20 parts per trillion) for each isotope](image)

Isotope conjugated antibodies have the same binding affinity as fluorophore tagged. Better resolution of isotopes offers multi-parameter assays regardless the marker expression (fair, abundant, negative). As example, it has been shown opportunity of
PBMCs analysis with simultaneous screening of 20 markers (Fig. 1.12 from Tanner et al, 2013 [195]). This opens new horizons in more confident diagnostics based on precise phenotyping.

**Fig. 1.12** Computer screen shot during mass cytometric analysis of adult PBMC. These cells were probed with antibodies against 27 surface antigens. Each antibody was labeled with a different stable isotope (given in the table at the top of the figure: the antigen is indicated, such as CD2, followed by the isotope used to tag the corresponding antibody, 175Lu). In addition, cellular DNA was labeled with an Ir-intercalator (used as a trigger for cell recognition).

### 1.7 Conclusion

This chapter gives an overview of quantum dots nature, properties, prospectives and current issues. One of the main challenges is lack of the instrumental methods suitable for precise quantification of nanoparticles on a single cell level, which would be able to deal with large cell populations. Numerous studies have showed toxic effects of engineered nanoparticles. The several strategies were developed to improve NPs biocompatibility. It is still little known about chemical and biological mechanisms of a cell-nanoparticle interactions. This review provides the state-of-art of nanotoxicity initiation, development and outcome to both cell and nanoparticle.
Objectives

The aim of presented work is to develop a reliable and simple multi-parameter method, which will allow to evaluate intracellular QD content and its effect on cell function. Flow cytometry was chosen for its quick sample preparation and processing, as well as opportunity to use multiple fluorescent channels in a single assay. This enables staining the cells with various biomarkers after cell exposure to the fluorescent nanoparticles. Also flow cytometry has an attractive function of subset sorting according to their fluorescence intensity. All together, it makes this technique a prominent candidate for express analysis of QD internalizing by live cells.

To summarize existing experimental results on QD cytotoxicity, we have built up a mathematical approach. It helps to predict the uptake kinetics and dynamics prior to an experiment. To date, it accumulated lots of experimental data of engineered material toxicity, but very few theoretical models. To fulfill it, we attempt to formulate a mathematical model which would let us to analyse cell fate depending on amount of ingested QDs.

In a number of studies, CdTe/CdSe quantum dots have been shown as potent inducers of oxidative stress, and, as result, cell death. It is believed, that leakage of toxic Cd$^{2+}$ is the main reason of toxicity. To address this issue, we investigated properties ternary semiconductor SbSI, which doesn’t contain heavy metals. We used different surfactants to control crystal formation and growth to obtain well-separated sub-microsized needles. As-prepared crystalline phases were tested on RAW264.7 monocytes for their biocompatibility.
Chapter 2. Materials and methods
2.1 Chemical methods

This section describes all chemical techniques which were used to synthesize and characterize CdTe and SbSI nano-scaled crystals. The initial set up, synthesis route, fractions separation and purification are fully described. As-prepared particles are characterised by UV-vis absorption, photoluminescence and electron microscopy.

2.1.1 Quantum Dots synthesis

Materials
Cadmium perchloride (CdClO₄) or cadmium chloride (CdCl₂) were taken as cadmium sources; aluminum telluride (Al₂Te₃) was taken as tellurium source. Sulfuric acid (H₂SO₄), thioglycolic acid TGA was used as stabilizer), sodium hydroxide (NaOH), gelatin and Millipore water were used as precursors in all synthesis. Propanol-2 (isopropanol) was used for final particles precipitation; Sephadex G 25 was used for size-exclusive, post-synthetic, purification. All listed chemicals were purchased from Sigma Aldrich, except Al₂Te₃. It was purchased from Cerac Inc. An Argon supply Schlenk line was used to purge the air, exhausting fume hood, 3-necked round bottom flasks, heating/stirring system, reflux setup, evaporator apparatus, pH-meter, balance, centrifuge, gel chromatography column, disposal polysterene cuvettes, 50 ml tubes, syringes, pipette tips and glass vials were used during the whole synthesis, purification and characterisation process. All precursors and consumables were kindly provided by Y. Gounko (Trinity College Dublin).

Method
CdTe quantum dots were synthesized according to previously published procedure [25]. Briefly, aluminum telluride was constantly stored under vacuum in order to avoid any oxidation. The required amount of Al₂Te₃ (approx. 0.5 g per each synthesis) was weighted and rapidly transferred into 3-necked flask under argon gas flow. Other precursors were measured according to the working molar ratio of 1:0.25:1.4 (CdCl₂:Al₂Te₃:TGA). The weighed Cd-containing salt (CdCl₂ in that particular case; however, CdClO₄ can be used as well) was dissolved in Millipore water under stirring.

A full synthesis setup is shown in Fig.2.1. Sulfuric acid was added in flask dropwise with Al₂Te₃; the resulted gas (H₂Te) was passed through a 3-necked round bottom flask (supplied with reflux and vigorous stirring) with CdCl₂ solution in 150 ml of Millipore
water. Then, TGA was poured to the reacting system; the clear solution became cloudy shortly afterwards. Two molar sodium hydroxide solution (NaOH) was added until initial transparent state of reaction mixture and pH adjusted to 11 (working value). Continuous heating and stirring was applied throughout synthesis. In the case of gelatin capped nanoparticles, 0.3 g gelatin was added as well. Afterwards, crystals were allowed to grow during the time required to enrich eligible size: as longer synthesis time – as larger their diameter.

After synthesis was completed, the primary transparent solution became turbid, reddish-brown, color, what indicated crystals formation. Then, the as-prepared colloid was evaporated by half of initial volume as it cooled down to 80°C.

Different size fractions were separated and collected as described:

1) The concentrated colloid was centrifuged for 10 min at 3000 rpm. The first fraction contains the biggest particles with significant diameter scatter. Thus it has low quantum yield and wide size distribution (as it's shown in Table 2.1)

2) The supernatant was harvested and mixed with propanol-2 due to generate the next fraction

3) Solid centrifuged sediment was dissolved in a minimal volume of Millipore water, suspended until became a homogeneous colloid, transferred in glass vial, marked and kept in fridge at 4°C

4) The precipitated second fraction was centrifuged, extracted, dissolved and stored as-described

5) All manipulations were repeated until supernatant has become pale yellow

6) All samples were stored at 4°C
Fig. 2.1 Synthesis setup. Cadmium containing salt is dissolved in water in presence of TGA. Aluminum telluride is reacted with sulfuric acid to produce gaseous tellurium hydride (H₂Te) and bubble it through Cd²⁺ solution. CdTe crystal seeds are formed under heating and constant stirring. They were let to grow into nano-crystals for a few hours, depending on eligible size. To protect system from contamination, argon gas is used.

**UV-vis and PL spectra**
The optical properties of the prepared colloids were measured on a Shimadzu UV-1601 UV-vis and Cary Eclipse PL spectrometer. The emission and absorption spectra were recorded, quantum yield was found using rhodamine 6 G as a reference; sample concentration, core diameter and number of particles were calculated. Results are shown in Table 2.1

*Table 2.1 List of physicochemical synthesized QDs properties*

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Coating structure</th>
<th>Absorption wavelength (nm)</th>
<th>Emission wavelength (nm)</th>
<th>Full width half peak of fluorescence (nm)</th>
<th>Quantum yield %</th>
<th>Concentration (mol/L)</th>
<th>Size (nm) ±0.1 nm</th>
<th>Number of particles (per ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VG 083 (1)</td>
<td>TGA-gelatin</td>
<td>535</td>
<td>588</td>
<td>56</td>
<td>1.00%</td>
<td>2.05E-004</td>
<td>3.1</td>
<td>1.23E+017</td>
</tr>
<tr>
<td>VG 083 (2)</td>
<td>TGA-gelatin</td>
<td>539</td>
<td>580</td>
<td>43</td>
<td>6.00%</td>
<td>3.32E-004</td>
<td>3.2</td>
<td>1.99E+017</td>
</tr>
</tbody>
</table>
Particles further storage and use
CdTe nanoparticles should be stored at 4°C in vials and covered by foil. For biological applications, in order to remove all traces of unreacted precursors, QDs should be purified in a gel chromatography size-exclusive column with Sephadex G 25 prior to exposure to the cells. Optical properties should be measured after purification.

2.1.2 SbSI synthesis and modification

Standard synthesis procedure
All precursors were purchased from Sigma Aldrich and used without further purification. Antimony trichloride (SbCl₃), sodium sulfide nonahydrate and potassium iodide, were weighed in proportion 1:1.2:1.4. SbCl₃ was dissolved in hydrochloric acid and placed into round-bottomed flask, which was inserted in ultrasonic water bath. The temperature was adjusted to 40°C. Na₂S and KI were dissolved in millipore water and added to the antimony (III) chloride. The orange precipitate (Sb₂S₃) was formed immediately, however it turned to deep red colour (SbSI) approximately after 15 min. The sonication time was 1 hour. Afterwards the solid product was decanted and washed in a hydrochloric acid followed by ethanol and milliQ water. The resultant dried powder was stored at room temperature; no alterations were observed over time.

Powder X-ray diffraction (XRD) structural analysis
As-prepared SbSI was examined for purity and crystal structure by XRD. The spectra were taken on Inel Equinox 6000 Powder X-Ray Diffractometer. The dried powder of
SbSI was placed into aluminium tab; the spectrum was taken in the 2theta range 10 to 80°. The recorded sample spectrum was compared to the standard. All characteristic peaks were in place; no impurities were found.

**Electron microscopy**
SEM described the morphology of obtained precipitates with EDX elemental analysis (Hitachi S-4700 Scanning Electron Microscope). TEM images were taken on Hitachi H7500 transmission electron microscope.

**Treatment with high power ultrasound**
In order to break down SbSI rosettes into individual nanoneedles and nanorods, the obtained powders were exposed to high power ultrasonication. Eighty mg of sulpho-iodide were dissolved in 80 ml of distilled water. Oleic acid and citric acid were used as stabilizers. The mixtures were treated with ultrasound for various times (from 10 to 80 min).

**Nano-needles optical properties test**
The resulted colored colloidal solutions were further examined for the absorbance and photoluminescence. The UV-vis spectra have been recorded on Shimadzu UV-1601 and fluorescence on Cary Eclipse spectrometers.

**2.2 Cell culture**
A range of cell lines and primary cells was used to examine the synthesized particles (SbSI and CdTe). RAW264.7 and U937 have monocyte morphology. RAW264.7 is adherent murine monocyte cell line. U937 is human monocyte cell line, which grows in suspension. MS-5 and NIH3T3 are murine connective tissue fibroblasts. Both fibroblast lines are adherent.

**Materials**
RAW264.7 and MS-5 cells were received from REMEDI, NUI Galway. NIH3T3 fibroblasts are the property of our group. U937 cells were provided by Dr. Alessandro Natoni (Apoptosis Research Centre, NUI Galway). The RAW264.7, MS-5 and NIH3T3
cells were cultured in T75 cell culture grade plastic flasks or well-plates. The disposable serological pipettes of 5, 10, 25 ml volume and pipette filler were used to manipulate the liquids. Fetal bovine serum (10%) and penicillin-streptomycin were used to complete the culture media. The cells were cultured in Dulbecco's Modified Eagle Media (DMEM), the 1X Trypsin (0.05%) and Hank's balanced salt solutions (HBSS) were used to sub-culture the monocytes. The RPMI-1640 media was used for U937 cells. Lonza serum-free media was used in primary cultures.

2.2.1 Cell Lines

RAW264.7
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% CO₂ at 37°C. Cells were regularly split into fresh cultures once the confluence reached 70%. A confluence is the percent of surface area occupied by cells. To remove the cells from substrate, a fluid wash technique was applied. Briefly, cells in a T75 flask were washed twice with 3-4 ml of sterile HBSS. Aliquot of the medium was pre-heated for 15 min in prior to experiment in water bath at 37°C. Fresh media was added to flask and vigorously pipetted up and down for 3-5 min. Bubbling and splashing were strictly avoided to reduce contamination and cell death. Harvested monocytes were seeded 1:3 or 1:4 ratio onto plastic substrates and placed back into incubator till further demand. The estimated seeding density is 1.5-2 million cells per flask.

MS-5 and 3T3 cell lines
The murine stromal fibroblast cell line MS-5 and NIH3T3 were used in the stent vascularisation study. The same media formulation as for RAW264.7 monocytes was used. Cells were passaged every 2-3 days depending on the confluence. To harvest the fibroblasts, cell were washed two times with 3-4 ml of sterile buffer (HBSS). Followed by washing, 2.5-3 ml of 1X Trypsin were added and gently spread over. The cells were trypsinised for 5 min in incubator. After that, the flask was slightly tapped to detach the cells. Equal volume of equilibrated to 37°C culture media were added to neutralise trypsin. Fibroblasts were centrifuged at 1500 rpm for 5 min. Supernatant was discarded;
the pellet was re-suspended in fresh culture media. Cells were split in 1:4 or 1:5 ratios, approximately 1 million cells in a T75 flask.

**U937 cell line**

Human leukemic monocyte lymphoma cell line was used for comparison with RAW264.7 murine monocytes. Unlike murine cell line, U937 grow in suspension. Cells were maintained in complete culture media (RPMI-1640, 10% FBS, 1% pen-strep), in a humidified atmosphere, with 5% CO₂ at 37°C. Cells were grown to a density of 2.5 x 10⁵ cells/ml in T75 flasks. Monocytes were passaged every 2-3 days. Cells were removed from incubator and centrifuged at 1500 rpm for 5 min, the supernatant was discarded and the pellet re-suspended in 5 ml of sterile HBSS. Cells in buffer were centrifuged and washing was repeated again. Afterwards cells were counted on haemocytometer and re-suspended in appropriate volume of fresh media.

### 2.2.2 Primary cells

**Periphery blood mononuclear cells (PBMCs) extraction and maintenance**

16 ml venous blood was collected from volunteer donors and proceeded immediately. Peripheral blood mononuclear cells (PBMCs) were extracted according to published procedure. Briefly, Lymphoprep buffer was overlayed with whole blood and centrifuged at 400 x g for 30 min. PBMCs band was harvested from buffer-plasma interface with a pasteur pipette and washed twice with HBSS. Cell pellet was resuspended in a complete cell culture media (X-VIVO 15 media) and seeded into a 24-well plate. After 75 min of incubation in humidified atmosphere at 37°C, 5% CO₂, non-adherent cells were removed; fresh cell culture medium was added and cells were grown further for 1 or 2 weeks. The media was changed every 2-3 days; cell confluence was regularly checked under light microscope.

The day before cytometry measurements, cells were removed from incubator, washed with HBSS. A fresh cell culture media containing QDs in eligible concentration was added into well plate. Cells were co-incubated with nanoparticles for next 24 hours. Afterwards, they were washed twice with serum-free PBS and harvested by pipetting. Cells were washed once with FACS buffer (PBS, 2% FBS, 0.1% sodium azide), stained with monoclonal antibodies (CD14, CD80) for 30 min at 4°C. The probes were proceeded to flow cytometry (BD FACSCanto II, 2 lasers, 8 colours instrument) at the
same day within 4 hours after staining. Untreated cells (did not receive any nanoparticles) and single stained samples were used as control panel. The FlowJo software v. 10 was used for statistical data analysis.

**Cell growth study on cardiovascular stents**
RAW264.7 monocites-macrophages were cultured according to protocol described above. Confluent T75 flasks were removed from incubator and cells were harvested by intense pipetting. The number of cells was estimated by counting on haemocytometer. The monocytes suspension was centrifuged at 1500 rpm for 5 min and the obtained pellet was re-suspended to achieve desirable density (approx. 2 million cells/ml). Concentrated cell suspension was added dropwise on the top of stent or flat sample. The samples were placed in incubator for 6 hours to let the monocytes adhere. Afterwards the samples were moved to fresh wells in well plate and fresh culture media was added. Cells on stents/flat probes were incubated overnight. The same protocol was applied to MS-5 fibroblasts.

**2.3 Electron microscopy**

**2.3.1 Transmission Electron Microscopy (TEM)**

**Cell culture**
RAW264.7 monocytes were routinely cultured as described. Cells were harvested according to standard procedure and seeded onto Thermanox films (13 mm diameter) in 24 well plate. The seeding density was 50,000 cells per well. The monocytes were incubated for 24 hours; after that, fresh media containing QDs in appropriate concentration replaced conditioned culture media. Cells were further incubated in presence of nanoparticles for next 12 or 24 hours as desired.

**Primary fixation**
Cells were fixed in 2 stages – through gluteraldehyde and osmication. The reduced culture medium was discarded; cells were washed twice with PBS followed by cacodylate buffer. After that, cells were primarily fixed with 2.5% gluteraldehyde, 2.5% PFA in sodium cacodylate buffer (25% gluteraldehyde, 10% PFA, sodium cacodylate in milliQ water) for 90 min. Then fixative was removed, cells washed twice with cacodylate buffer.
Secondary fixation
Next, cells were processed to osmication – secondary fixation in 1% osmium solution. It is necessary step to increase the contrast of TEM images. The samples were introduced to the osmium solution for 60 min. Used osmium solution was appropriately discarded; used Pasteur pipettes were placed in 2% ascorbic acid solution to neutralise osmium.

Dehydration
Samples were introduced to ethanol gradient to dehydrate them. The following procedure was applied:
- 50% EtOH for 15 min at room temperature
- 70% EtOH for 15 min at room temperature
- 90% EtOH for 15 min at room temperature
- 95% EtOH for 15 min at room temperature
- 100% EtOH for 15 min at room temperature

Resin embedding
Followed by ethanol gradient, samples were introduced to low viscosity resin gradient made according to recommended protocol:
1. 1 hour 25% resin and 75% EtOH at room temperature
2. 1 hour 50% resin and 50% EtOH at room temperature
3. 1 hour 75% resin and 25% EtOH at room temperature

Cells sections and imaging
Afterwards thermanox films were moved to silicon moulds and filled with 100% resin. For resin hardening, the moulds were baked at 65°C for 72 hours. The thermanox film was peeled off; the hard blocks were cut and trimmed to produce thin sections (approx. 90 nm thick). The sectioned cells were placed onto copper TEM grids. No post-fixation has been performed to avoid artefacts, which can be confused with nanoparticles.
2.3.2 Scanning Electron Microscopy (SEM)

**Fixation**
After incubation, the stents/flat probes were removed from incubator. The reduced culture media was carefully removed from the wells. The samples were gently washed twice with non-sterile PBS. Cells were fixed in 2.5% gluteraldehyde, 2.5% PFA mixture overnight at room temperature. Fixative was obtained from 25% gluteraldehyde, 4% PFA in PBS and non-sterile PBS.

**Dehydration**
The fixative was gently discarded from the wells; samples were washed twice with non-sterile PBS. The ethanol gradient has been introduced to dehydrate the cells as following:

1. 50% EtOH for 5 min at 4°C
2. 75% EtOH for 5 min at 4°C
3. 80% EtOH for 5 min at 4°C
4. 90% EtOH for 5 min at 4°C
5. 100% EtOH for 5 min at 4°C

The dehydrated samples were stored in absolute ethanol (100%) till next step. Eligibly they were immediately processed to drying.

**Drying**
The samples were dried by critical point drying (CPD) technique. Briefly, CO₂ was flown through the CPD chamber to bring the temperature down to 4°C. Once it was cooled down, CO₂ was turned off, the chamber was filled with absolute ethanol and samples were placed in chamber. The meniscus level was checked; the chamber was pressurised. The heating was switched on and left till temperature had reached 32°C and pressure was maintained at 1250 psi. Afterwards heating was stopped and pressure was gently released. The dried samples were removed from the chamber, adhered to carbon tabs and coated with gold. Afterwards samples were stored in dried place (sealed Petri dishes filled with silica granules to absorb water from the air) until viewie under scanning electron microscope.
2.4 Biological in vitro assays

2.4.1 Live/Dead Assay
Live/Dead Assay (Life Technologies) was used to visualize viable and necrotic cells. Cells were seeded onto a 8-well glass bottomed chamber in density 50,000 cells per well and cells were incubated for 24 hours. Next day, the appropriate amounts of QDs were added into the chamber and cells were placed back in incubator for 12 or 24 hours, respectively. After co-incubation, samples were washed twice with PBS and stained with calcein and ethidium bromide (EthD-1) from the kit as recommended by manufacturer. Briefly, 2 uM calcein and 4 uM EthD-1 stock solution was made up in PBS at the day of assay. 400 uL of the stock were added to cells, the chambers were covered up with foil and incubated at room temperature for 45 min. Slides were proceeded within an hour for fluorescent microscopy.

2.4.2 Fluorescent microscopy
Cell morphology was tested at each time point. Actin was stained with phalloidin eFluor 760 (eBiosciences) according to recommended procedure. Cells were also seeded at density of 50,000 per well in 4-well chamber slide and let grow overnight. Next day, the QD solution was added to the slides and incubated for further 12 or 24 hours. Untreated monocytes were used as control. Afterwards cells were removed from incubator, washed with PBS and fixed with 4% PFA for 15 minutes. The fixed cells were permeabilised with 0.2% Triton X solution for 5 min, washed with PBS and stained with phalloidin for 1 hour. DAPI solution was added to stain nuclei; the slides were viewed immediately under inverted fluorescent microscope.

2.4.3 Alamar Blue
To examine cell metabolic activity, Alamar Blue® assay has been used. The cells were seeded in a 24-well plate to a density of 1x10^5 cells per well, 24 hours prior to experiment. Different types of QDs (either TGA or TGA-gelatin-covered) within a range of concentrations (1 – 100 nM final concentration) were added to macrophages. After 12 or 24 hours of co-incubation, the cells were progressed to Alamar Blue assay according to protocol. Conditioned culture media was discarded, the cells washed twice with PBS. Cells were treated with 10% Alamar Blue solution and incubated for 4 hours
at 37°C, 5% CO2, humidified atmosphere. After that, cells were removed from incubator and dye reduction was analysed by fluorometric reading on Varioskan Flash plate reader at 570 nm excitation and 610 nm emission wavelength.

2.4.4 ds-DNA Quantification
Quant-iT Pico Green ds-DNA Assay Kit was used for a precise counting cell number in the probe. The cells were seeded in a 24-well plate to a density of 1x10^5 cells per well, 24 hours prior to experiment. Different types of QDs (either TGA or TGA-gelatin-covered) within a range of concentrations (1 – 100 nM final concentration) were added to macrophages. After 12 or 24 hours of co-incubation, the cells were progressed to PicoGreen assay according to protocol. Conditioned culture media was discarded, cells washed twice with PBS. To release DNA, 250 ul of milliQ water were added to each well and cells underwent repeated (3 times) freezing-thawing cycle at -80°C/room temperature. The DNA content was analysed by fluorometric reading on Varioskan Flash plate reader at 485 nm excitation and 528 nm emission wavelength.

2.5 QDs uptake study by flow cytometry
2.5.1 Principles of the method
Flow cytometry is a physical method what allows analysing multiple characteristics of a single cell for a large number of cells. It measures optical properties, such as cell size (forward light scatter), intracellular complexity/density (side light scatter); it helps to recognise and target different cell populations (e.g. lymphocytes, granulocytes, erythrocytes in whole blood). Various fluorochromes can be tagged to wide range of surface molecules or cellular compartments (DNA, RNA) and proteins. To avoid signal irregularities, the injected sample should be single cell suspension; besides big aggregates can clog and damage the machine.

When the labelled cell passes the interrogation point, a laser excites the attached fluorescent molecule to a higher energy state. The molecule emits photons, which are registered by the light detectors; the fitted filters separate the signal into narrow wavelength bands. The resulted bands can be converted into electronic signal and quantified in special software. Depending on cytometer type, up to 8 fluorescent channels can be used simultaneously.

The panel of appropriate controls should accompany any flow cytometry measurements;
first of all, the fluorescence from unstained cells should be recorded for further voltage adjustments and gating on target populations. Secondly, single stained probes (or isotype controls) should be included in experiment to check staining index. If multi-colour panel with interfering fluorophores is used in an experiment, we're facing to spectral overlap problem. In the case when both markers co-expressed by the same cell and two dyes have close emission spectra, it creates high background in both channels. This effect is called a spillover; the width of spillover is a linear function, thus it can be resolved by applying simple linear algebra. This mathematical operation is called compensation. If there are more than 2 overlapping channels, compensation must be performed for each pair.

2.5.2 Sample preparation for flow cytometry
Monocytes were cultured in 6-well plates and treated with nanoparticles as described above. On the day of flow cytometry measurements, the well plates were removed from incubator and placed in fume hood. The reduced culture media was removed and cells were washed twice with HBSS. Fresh culture media was added to the well plates and cells were harvested by vigorous pipetting. RAW264.7 cell line appeared to be resistant to Trypsin; scrapping technique was also found ineffective due to massive cell damage. Thus pipetting with 1000 ul micropipette was used in all flow cytometry experiments. The obtained cell suspension was placed in marked Eppendorf tubes and centrifuged at 1200 rpm at 4°C for 5 min. The supernatant was discarded, the pellet re-suspended in PBS and centrifuged. The cells were washed till media traces were fully removed. After that, cells were washed twice in serum containing FACS buffer: PBS, 2% FBS, 0.1% sodium azide. The prepared buffer is kept refrigerated. In a basic experiment, when no additional labelling was involved, the cell pellet was re-suspended in 500 ul FACS buffer and proceeded to flow cytometry immediately. In between operations tubes with the samples were kept on ice.

2.5.3 Gating strategy
In each set of measurements performed, the first tube contained just untreated monocytes, without any fluorescent tags/nanoparticles. This gives us baseline values in the reference channels and further used for detection of appropriate cell population.
Prior to a first run, the forward and side light scatter were adjusted according to values recommended by technician. To exclude any mechanical debris, threshold value was adjusted to 5,000 events. A firm, consistent cell population is gated as it shown on Fig. 2.2A. The selected subset was plotted in FITC/APC channel as histogram to establish background. For correct QDs detection, the voltage in FITC (green QDs) and APC (red QDs) channels was adjusted to give us small, but detectable value. Typically, at least 10,000 events were recorded. After the correct gates and voltage were found, no further changes were made. The probes of cells containing QDs were ran, the result is represented as peak shift (Fig. 2B). In-built statistics options were used to interpret results.

![Image](Fig. 2.2 Basic gating strategy for QD uptake evaluation. Primary gates (A) are chosen based on physical parameters of the cells (size/internal consistence). Afterwards cell population was examined on fluorescent response in QD reference channel (B). Peak shift to the right corresponds to increase in intracellular fluorescence from cells treated with 100 nM QDs (red line). Three coinciding peaks belong to control, 1 nM, 10 nM QDs and refers to no uptake in case of low doses.)

2.5.4 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. Briefly, the reduced
media was removed and the cells were washed twice with phosphate buffered saline (PBS). Macrophages were harvested by pipetting in fresh media and then were placed in eppendorf tubes. Cells were washed twice with PBS immediately after harvesting, re-suspended in 500 ul 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 processed 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.

2.5.5 QDs uptake and CD80/86 surface markers expression
Flow cytometry was used to detect the amount of internalized nanoparticles and to measure the expression of pro-inflammatory receptors caused by exposure to QDs. All measurements were performed on BD FACS Canto A. In this experiment cells were seeded into 6-well plates to a density of 2.5x10^5 cells per well and left 24 hours to adhere. The next day, macrophages were loaded with different types of QDs within a range of concentrations (1 – 100 nM final concentration). After 12 hours of treatment (for the CD86 study) and 24 hours (for the CD80 study), the probes were processed to the assay according to a standard protocol. Cell were harvested and washed twice in PBS followed by 2 times washing in FACS buffer (PBS, 2% FBS, 0.1% sodium azide). Armenian hamster IgG and Rat IgG2a K were used as isotype controls for CD80 and CD86, respectively. All antibodies and isotype controls were purchased from BioLegend. The standard staining protocol recommended by manufacturer was employed. Cells were re-suspended in FACS buffer, appropriate volume of antibodies was added. Samples were incubated at 4°C at least for 30 min. Cells were centrifuged at 4°C, washed 2-3 times in FACS buffer. Afterwards the cell pellets were re-suspended in 500 ul of FACS buffer and processed to flow cytometry. APC and FITC channels were used as references for signal detection. FlowJo software was used for results interpretation.
2.5.6 Intracellular quantification of QDs
The amount of ingested nanocrystals was defined by FlowJo software. At least 10,000 events were recorded per tube. Consistent macrophage population was selected from light scatter graph, the levels of fluorescence in FITC and APC channels (for green and red particles, respectively) were 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 control in the reference channel.

2.6 Conclusion
A wide range of experimental techniques was used in this work. This chapter reviews methods of nanoparticle synthesis, characterisation and in vitro testing. The nanostructers of two different semiconductors – binary and ternary – were fully described. The optical properties of the obtained fractions were examined for UV-vis absorption and fluorescence. The size and morphology of crystals were described by SEM, TEM and XRD (for SbSI only).

The cell function and fate were investigated after the exposure to a number of biomaterials with various surface structures and compositions. The bioassays include Alamar Blue, PicoGreen, Annexin V, Live/Dead and surface markers profiling. Fluorescent, light and electron microscopy were routinely applied to the samples acquisition.
Chapter 3. Overview
Fluorescent nanocrystals are made of semiconductor compounds are called Quantum Dots (QDs). These nanomaterials were first synthesized and named by M. Reed in 1985 [16]. Since that time various uses of QDs have been developed including their applications in photonics, energy harvesting and bio-imaging. Unlike organic fluorochromes, the optical properties of QDs include a large Stokes shift, broad absorption and narrow emission, bright fluorescence and high resistance to photobleaching. The set of unique size-tuneable optical characteristics, ease of manufacturing, surface modification and bioconjugation made them eligible alternates for organic dyes as fluorescent agents [196]. Despite the benefits provided by QDs, the challenge of quantifying altered intracellular components is complicated, and not clearly investigated, due to interaction of nanoparticles with different cellular compartments. The issues of cyto- and nano-toxicity and the lack of simple reliable methods for quantification of ingested QDs hamper their broad integration in in vivo and clinical imaging applications. Several techniques were employed to resolve this problem, however they all suffer from significant disadvantages. Table 3.1 summarizes the strong and weak sides of major quantification techniques. Confocal and fluorescent microscopy methods are widely applied for complex multi-parameter cell assay. Due to fact that cells are stained with organic fluorochromes, it limits imaging time. Also the cells must be cultured in special chambers with certain optical properties. The main weakness of the methods is high dependence on instrument calibration, which affects result reproducibility. Image analysis is the tool used for statistical data analysis and interpretation for any microscopy technique. Electron microscopy has excellent resolution, which allows visualizing organelles morphology and does not require use of fluorophores. It makes possible to quantify the uptake of nanoparticles [197], but it is time consuming and does not reflect processes taking place on realistic cell population. Also it operates with very limited cell number (<100). Inductively coupled plasma mass spectrometry (ICP-MS) is relatively straightforward technique employed by several groups [198-200]. It allows precise measure of cadmium in the probe even on single-cell level [201]. It does not specify a nanoparticles location or any structural changes. Flow cytometry is a perfect tool to describe cell population behaviour based on signal harvested from individual cells. In a combination with mass spectroscopy it gives a unique opportunity to perform unbiased quantitative assay and evaluate exact number of QDs inside the cells in population of interest. A major part of the work was aimed to investigate the technique capability as QD quantification method.
<table>
<thead>
<tr>
<th>Method</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light microscopy</td>
<td>Fast; doesn’t affect cell viability; cells can be cultured further. Large number of cells can be visualised</td>
<td>Gives information only about number of cells and can confirm only absence of morphological changes. Only available low magnification option</td>
</tr>
<tr>
<td>Fluorescent/Confocal microscopy</td>
<td>Can visualise QDs; able to distinguish intracellular localisation and assess various organelles integrity</td>
<td>Can visualize limited number of cells; great variability between images taken at different days; requires expensive glass-bottom culture dish</td>
</tr>
<tr>
<td>Electron microscopy</td>
<td>Can directly visualize nanoparticles with very high magnification and resolution; possible high resolution 3D reconstruction of the cell; Gives detailed information on cell morphology; QDs can be quantified on single cell level</td>
<td>Time consuming (takes up to a week to prepare the final sections); operates with only few cells (less than 100); Sample preparation is also technically complicated</td>
</tr>
<tr>
<td>Mass spectrometry</td>
<td>Provides exact information on intracellular elemental content of QD; easy to reproduce and compare results; simple instrument calibrate; Able to work with large number of cells</td>
<td>Can analyse only overall amount of nanoparticles; completely destroys the cells; doesn’t give any information about cell morphology or viability</td>
</tr>
<tr>
<td>Mass cytometry</td>
<td>Has all advantages of mass spectrometry; also able to</td>
<td>The instrument is expensive and rarely</td>
</tr>
</tbody>
</table>

Table 3.1 Overview of main QD quantification methods
analyse single cells; other cellular parameters can be acquired simultaneously with QD content; large number of parameters can be analysed at the same time

| Flow cytometry | Able to analyse cell morphology and cell function along with QD quantification; fast, easy to calibrate; simple way to analyse the data; instrument is widely available, low cost of consumables; easy calibration strategy; can process large number of cells (>10,000) | Number of parameters is limited by application of molecular dyes; if multiple colours are used, fluorescence compensation is required; Doesn’t provide information on exact number of QDs inside the cells; |

QDs are easy to detect in concentrations of 100 nM when exposed to phagocytic cell cultures for 12-24 hours. Lower concentrations (1 and 10 nM) did not result in any visible fluorescent response. The nanoparticles uptake was described in conjunction with a number of cellular responses (pro-inflammatory markers expression, cell viability, death mechanism). Four different types of particles were explored with core sizes ranging from 2.1 to 5.3 nm and either TGA or TGA-gelatin coating. Unlike the proposed model by Chithrani and Chan [61], QD uptake dynamics and kinetics are strongly depend on particle size.

As mentioned previously, flow cytometry has been shown as convenient and relatively fast method for semi-quantitative analysis of fluorescent nanoparticle uptake and their effect on cell function on cell population level. This is a significant benefit as other more accurate methods are both too slow in sample preparation and processing or deal only with average values and not individual cells.

Green TGA-capped nanoparticles, with a core size as small as 2.1 nm, have been investigated. The general tendency of small green-emitting QDs to penetrate cells and
actively interact with organelles in aggressive manner was suggested by Lovric et al, 2005 [42] and confirmed in our experiments. Such behaviour is explained by uptake kinetics which can be described by “fast model” for green TGA-capped QDs – due to a lower number of attached albumin molecules and smaller eventual hydrodynamic radius. These QDs are more easily trapped and destroyed by macrophages, which results in rapid nanoparticles clumping and leaching of cadmium ions, hence higher toxicity. Apoptosis was found as the main scenario for cellular fate after exposure to QDs; the late apoptotic subset was significantly larger than early apoptotic, which was nearly absent in most probes. Late apoptotic cells are shown as brightest upon others due to their higher density. Several apoptosis hallmarks were detected by electron microscopy, including breach of mitochondria membrane, swelling of organelles, condensation of chromatin etc.

Incorporation of QDs in inert outer shell has been proved as efficient strategy to reduce ineligible cytotoxicity. In the current work gelatin is explored as a cell-friendly isolating material, as it contains hydrolysed fragments of various proteins, mainly collagen. Our experiments showed that gelatination has increased QD intracellular retaining time. Indeed, monocytes accumulated nanoparticles over time, this resulted in a significant increase of fluorescent response. It also leads to induction of cell death. According to our results, the uptake has reached nearly 100% at 24 hours acquisition point.

In summary, was developed a simple approach for semi-quantitative intracellular QD quantification. Compared to other methods, it is fast, gives statistically reliable results, able to handle big cell populations and allows carrying out other multi-parametrical bioassays at the same time. However, this method can’t detect the exact intracellular localisation or amount of. To our best knowledge, available to-date precise quantification methods deal only with particles at least 40-50 nm in core size, whereas we considered QDs in size range 2.1-5.3 nm. Such drastic dimensional difference gives rise to completely new challenges and affects compared to larger species. Thus new instrumentation and technology is required for further investigations. For example, in last few years has been successfully developed a crossover technique between mass spectroscopy and flow cytometry – mass cytometry. It is unique technology, which allows quantifying exact amount of the chemical elements inside individual cells or use heavy isotopes to tag biomarkers and antibodies. It can be an effective strategy to evaluate precisely the intracellular content of the QDs and enlighten the mechanisms behind their toxicity and interaction with cellular compartments. The main issues are
limited availability of the equipment, high costs, complicated operation and service. Based on obtained experimental results, we have attempted to build up a mathematical model of QD cytotoxicity. For the first approach, number assumptions has been made 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.
- 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.
- Apoptosis and necrosis are irreversible processes, thus once a cell has entered one of the pathways, it’s resulted in cell death.

The model is composed of ordinary differential equations that tracks the evolution over time of four cell subpopulations, namely: healthy cells, apoptotic cells, necrotic cells, and dead cells. The aim of the modelling is to quantitatively assess the response of a population of healthy cells to exposure from quantum dots. This is a simple type of mathematical model, however it is universal and can be applied to describe similar systems dealing with species of the same size range (some toxins, bacteria). The suggested model can be significantly strengthen if intracellular QD concentration is known. That means mass cytometry would be the best option to obtain the data required. Also more detailed experimental measurements will enable formulation of predicting models rather than descriptive. It is also significant, that most of effective models are developed for non-toxic (polymer, silver, gold) nanoparticles 40-50 nm size range. Such model, as developed in our work, is an important step to prediciting theoretical nanotoxicity. There is a new type of nanoparticles being synthesized every day; thus it is not feasible to investigate all possible effects experimentally. That’s why establishing valid mathematical models is a necessity dictated by nanoscience.

Even after two decades of intensive development, binary QDs still have many issues. Apart from mentioned toxicity, they have very weak emission in near-infra red spectrum (NIR), which is optimal for deep tissue imaging. The capacity of penetration through live tissues without signal scatter is vital for non-invasive techniques, which have clinical potential. It led to an idea to develop new type of QDs composed from
ternary semiconductors, well known for their piezoelectric and interesting optic effects. As part of the project, we have explored properties of antimony sulpho-iodide (SbSI) as promising candidate for NIR QDs. This material has needle morphology and set of exceptional optic characteristics, however it is hard to synthesize in controlled manner. We have explored the factors, which influence crystal growth kinetics and obtained SbSI needles and rods. As-prepared phases have high crystallinity and purity. Cytotoxicity tests have showed strong ability of the material to kill quickly growing cells, what can be potentially used in anti-cancer therapy, especially due to fact that SbSI is pyro-optic material, i.e it tranduces light energy into the heat.

To expand our knowledge in other types of biomaterials, a collaboration project has been conducted. We have tested cell attachement and growth onto cardiovascular stents with different surface texture. The surface ripples were introduced by laser pulse shots; two different wavelength were used to regulate ripple depth. Afterwards, the stents were introduced to monocyte and fibroblast cell cultures as substrates. The cell adherence and growth were examined by scanning electron microscopy (SEM). Bare (untextured) stents and samples treated with culture media only served as controls. As results, has been observed distinct and consistent reaction on the generated ripples. Monocyte cultures, which belong to circulating immune cells, did not adhere to any rippled samples, but preferred smooth surfaces instead. In contrast, fibroblasts, which represent connective tissue, have demonstrated clear affinity to rippled stents; they failed to attach to bare samples. Interestingly, when the same set of experiments has been conducted on flat samples, all cell types have made a strong connection. It highlights an importance of in vitro tests on actual biomedical device design rather than coupon material, which doesn’t reflect the final topology. It is not a trivial observation, as many biomaterial studies have been done on flat samples; however, these results can not be translated any further.

**Conclusion to the published papers**

Contribution: experiment design, execution, data analysis, manuscript preparation and publishing

Contribution: biological experiments design, cell culture on stents, sample preparation, data analysis, participation in manuscript writing and processing

Contribution: experiment design, supervision of synthesis and further description of the material, biological tests design and execution, data analysis, manuscript preparation and publishing.

Contribution: experimental part design and execution (biological tests of QDs in cells), data analysis, model review, manuscript writing (equally with Paul Greaney).

5. Fernandez-Yague, Marc A.; Larrañaga, Aitor; Gladkovskaya, Olga; Stanley, Alanna; Tadayyon, Ghazal; Guo, Yina; Sarasua, Jose-Ramon; Tofail, Syed; Zeugolis, Dimitrios; Pandit, Abhay; Biggs, Manus. The effects of Polydopamine Functionalization on Boron Nitride Nanotube Dispersion and Cytocompatibility. ACS Bioconjugate Chemistry.
Contribution: cytotoxicity tests design, execution and supervision, data analysis, participation in manuscript writing and processing

Contribution: experiment design, execution, data analysis, manuscript preparation and publishing
References

18. Di, W., et al., Single-phased luminescent mesoporous nanoparticles for
37. Jamieson, T., et al., *Biological applications of quantum dots*. Biomaterials,
76


187. Рыбина, И.Н., Синтез и свойства ряда серосодержащих пьезоэлектрических материалов; диссертация ... кандидата технических наук : 05.17.01 1997.


Chapter 4. Published peer-reviewed papers
The interaction of QDs with RAW264.7 cells: nanoparticle quantification, uptake kinetics and immune responses study†

O. Gladkovskayaab, V. A. Gerard,c M. Nosovc, Y. K. Gun’ko, c G. M. O’Connora and Y. Rochec††

Fluorescent semiconductor nanocrystals called quantum dots (QDs) have been proposed as a prominent bio-imaging tool due to their exceptional optical properties. Typically the core size is not greater than 10 nm, thus QDs don’t obey models successfully developed and proved on practice for large particles (40–200 nm). This makes it difficult to predict the behaviour of such small yet reactive species in physiological media. Despite the benefits provided by QDs, the challenge of quantifying altered intracellular components remains complicated, and is not clearly investigated, due to interaction of nanoparticles with different cellular compartments. The goal of this work is to investigate uptake kinetics of small green-emitting TGA-capped CdTe QDs with diameter as small as 2.1 nm and to quantify their accumulation inside the cells over the time by flow cytometry. The effect on RAW264.7 monocyte–macrophage cell function and viability also was studied, as monocytes play an important role in innate immunity. The optimal parameters (QD concentration, exposure time, cell activation status) were found; the tested nanoparticles are proven to be applied in short-term assays due to their quick ingestion and accumulation.

1. Introduction

Nano-sized particles of well-known bulk materials (such as silica, carbon, titanium dioxide, etc.) have enabled many unique possibilities in different technologies and disrupted existing technologies. Engineered nanoparticles are poised to make key impacts in many biological and medical applications, like controllable drug delivery and release systems, gene diagnostics, and bio-imaging. The question of how these developments can be applied safely in humans remains open. Fluorescent nanocrystals made of semiconductor compounds are called Quantum Dots (QDs). These nanomaterials were first synthesized and named by M. Reed in 1985. Since that time various uses of QDs have been developed including their applications in photonics, energy harvesting and bio-imaging. Unlike organic fluorochromes, the optical properties of QDs include large Stokes shift, broad absorption and narrow emission, bright fluorescence and high resistance to photo-bleaching. The set of unique size-tuneable optical characteristics, ease of manufacturing, surface modification and bioconjugation made them eligible alternates for organic dyes as fluorescent agents. However, the discovery of new molecular fluorescent tags and their alternates is under extensive research. For example, steady fluorescent response with good Stokes shift and target mRNA binding has been achieved bio-constructs with perylene-2’-amino-4’NA as fluorescence reporter. The vast absorption profile of QDs allows use of a non-specific light source. As shown in Fig. 1, green QDs can be excited by either a violet or a blue laser. In contrast, molecular fluorophores for a maximum efficiency require excitation at a specific wavelength, which is often difficult to achieve because a cytomter is usually fitted with only 2 or 3 lasers. The spectral overlap, which typical

Fig. 1 Possible range of available lasers (vertical lines) and UV-vis absorption of green-emitting QDs used in the study. Due to broad absorption profile QDs don’t require excitation on specific wavelength.
for organic dyes limits the number of colours that can be used in single assay; QDs are not limited by this effect.

Fluorescent proteins (FPs) are great genetic labels which have an option to be in-built into target. Being assembled within a cell, they don’t require further fixing/permeabilising or any other cell intervention by exogenous agents. This class of fluorescent tools has been widely explored in live in vivo and in vitro imaging, and fundamental researches including protein ageing, localisation, morphology etc. The history and application of FPs is excellently described in review by Chudakov and papers by Krems and Chen. Unless exceptional set of properties provided, FPs have few weak points: (1) large size (25 kDa), whereas molecular fluorophores are just 1 kDa in average; (2) extreme susceptibility to media conditions; even minor pH fluctuations are able to impair FP stability and hence optical properties.

Quantum Dots are shown as prospective fluorescent tags in a range of bio-conjugates, including anti-cancer antibodies, drugs and receptors. More details about in vitro and in vivo targeting, delivery and imaging can be found in reviews. Molecular Beacons (MBs) technology is a powerful tool in live bio-imaging, disease diagnostics and molecular recognition. Based on the biochemical principle of selective complementary nucleobases binding, MBs enable single-base DNA mismatch detection. It plays a key role in mutations and other pathological alterations detection. Upon binding of complementary sequence, MB opens and thus release a fluoroophore. Subsequently, basic hairpin approach has been enormously improved and fitted to different ways of application and recently, such kind of technique is widely implemented in real-time PCR monitoring, developing DNA sensors, investigations of gene activity, bio-imaging and cancer targeting, DNA-protein interactions. However, the highest FRET response level and signal-to-noise ratio were achieved using semiconductor QDs as fluorescent moiety. In our group we have carried out the grafting of molecular beacons to QDs. Obtained hybridized nanoprobe have demonstrated improved optical characteristics, absence of background noise and high affinity to chosen target (data not published).

The inherent function of macrophages is to engulf species recognized as “non-self”, such as dead cell debris and bacteria. Macrophage cell response is the first line in adaptive immunity, their surface has a number of markers susceptible to both toll-like receptors (TLR) and mannose receptors. Another category of surface proteins is responsible for triggering inflammation cascades by expressing inflammatory interleukins (IL), chemokines, cytokines, reactive oxygen species (ROS), nitrite oxide (NO) and cyclooxygenases (COX). Macrophage cells are a convenient in vitro model for investigations on QD endocytosis and their further tracking due to quick ingestion within the time scale of live bio-imaging (within a few hours). It can help evaluate all the reactions correctly for objective results to be realised regarding particle efficacy and toxicity.

The main purpose of this work is to describe the uptake kinetics of small nanoparticles (2.1 nm) over the time; also we aimed to develop a simple method for quantum dot intracellular quantification and to investigate QD behaviour at different levels of interaction in physiological media conditions. Particles toxicity, intracellular fluorescence, inflammatory markers expression and cell death mechanism were investigated at 12 and 24 hours time points. Flow cytometry was used to measure cellular responses and quantify nanoparticle ingestion at a specific population level.

2. Materials and methods
2.1 QDs synthesis
CdTe QDs were synthesised according to a previously published procedure. Briefly, Al₂Te₃ reacted with sulphuric acid to produce H₃Te gas which was bubbled through an aqueous solution of CdCl₂, thiolglycolic acid (TGA) and 0.3 g of gelatin, with pH buffered at 11. The molar ratio of Cd : Te : TGA was 1 : 0.25 : 1.4. The reaction mixture was then heated under reflux for 2 to 48 hours depending of the desired nanoparticle size. Narrow size distribution fractions were collected via size-selective precipitation using isopropanol.

2.2 UV-vis and PL spectra
Absorbance was examined on a Shimadzu UV-1601 spectrophotometer; distilled water was taken as a baseline. PL spectra were recorded on a Cary Eclipse spectrometer. All measurements were performed to characterize the optical properties of the nanoparticles obtained. More detailed description of as-prepared QDs can be found in the papers previously published by our group.

2.3 Cell culture
RAW264.7 murine macrophages 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⁻¹ of penicillin and 100 µg mL⁻¹ of streptomycin. Macrophages were maintained in a humidified atmosphere with 5% CO₂ at 37 °C.

2.4 Fluorescent microscopy
Cell morphology was tested at each time point. Actin was stained with phalloidin eFluor 760 (ebioscience) according to recommended procedure. Cells were seeded in density 50 000 per well in 4-well chamber slide and let grow overnight. Next day QDs solution was added to the slides and incubated for further 12 or 24 hours. Untreated monocytes were used as control. Afterwards cells were removed from incubator, washed with PBS and fixed with 4% PFA for 15 minutes. Fixed cells were permeabilised with 0.2% TritonX solution for 5 min, washed with PBS and stained with phalloidin for 1 hour. DAPI solution was added to stain nuclei; the slides were viewed immediately under inverted fluorescent microscope.

Live/Dead Assay (Life Technologies) was used to visualize viable and necrotic cells. Cells were treated with QDs as described above. After co-incubation, samples were washed with PBS and stained with calcine and ethidium bromide from the kit as recommended by manufacturer. Slides were proceeded within an hour for fluorescent microscopy.
2.5 Double stranded-DNA (ds-DNA) quantification

Quant-it PicoGreen ds-DNA Assay Kit was used for a precise counting cell number in the probe. The cells were seeded in a 24-well plate to a density of 1 x 10^5 cells per well, 24 hours prior to experiment. Different types of QDs (either TGA or TGA-gelatin-covered) within a range of concentrations (1-100 nM final concentration) were added to macrophages. After 24 hours of co-incubation, the cells were progressed to PicoGreen assay according to protocol.

2.6 Annexin V apoptosis assay

In this assay cells were seeded to a density of 2.5 x 10^5 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. Briefly, the reduced media was removed and the cells were washed twice with phosphate buffered saline (PBS). Macrophages were harvested by pipetting in fresh media and then were placed in Eppendorf tubes. Cells were washed twice with PBS immediately after harvesting, re-suspended in 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.

2.7 QDs uptake and CD80/86 surface markers expression

Flow cytometry was used to detect the amount of internalized nanoparticles and to measure the expression of pro-inflammatory receptors caused by exposure to QDs. All measurements were performed on BD FACS Canto A. In this experiment cells were seeded into 6-well plates to a density of 2.5 x 10^5 cells per well and left 24 hours to adhere. The next day, macrophages were loaded with QDs within a range of concentrations (1-100 nM final concentration). After 12 hours of treatment (for the CD80 study) and 24 hours (for the CD86 study), the probes were proceeded to the assay according to a standard protocol. Armenian hamster IgG and Rat IgG2a K were used as isotype controls for CD80 and CD86, respectively. All antibodies and isotype controls were purchased from BioLegend. The standard staining protocol recommended by manufacturer was employed. APC (Allophycocyanin) and FITC (Fluorescein isothiocyanate) channels were used as references for signal detection. FlowJo software was used for interpretation of results.

2.8 PMA activation and CD86 expression study

Phorbol 12-myristate 13-acetate (PMA) was used to activate monocytes as described elsewhere. Cell cultures were prepared as described above. Cell culture media was supplemented with 100 ng mL^-1 of PMA and monocytes were conditioned for 6 hours. Afterwards PMA containing media was replaced by QD solution in normal media. The cells were co-incubated with nanoparticles for 12 hours and proceeded CD86 assay as in previous section. Unprimed monocytes, cells treated only with PMA or QDs and isotype stain were used as controls.

2.9 Quantification of QDs

The amount of ingested nano-crystals was defined by FlowJo software. At least 10,000 events were recorded per tube. Consistent macrophage population was selected from light scatter graph, the level of fluorescence in 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.

3. Results

3.1 PicoGreen assay

Fig. 2 presents the results of ds-DNA quantification taken at 24 hours co-culture. Only 100 nM concentration significantly reduces the number of viable cells (either due to necrosis or apoptosis). The inert reaction on 1 and 10 nM can be explained by the threshold effect: a certain critical concentration of particles in system should be achieved to trigger ingestion. To prove it, independent flow cytometry measurements were taken to evaluate intracellular amount of QDs.

3.2 Cell morphology and Live/Dead assay

Monocytes demonstrate healthy round morphology, actin is uniformly spread compactly around cells without any disruption in samples with QDs concentration less than 100 nM at both time points. Exposure to high concentration of nanoparticles leads to significant reduction cell number. After 24 hours of treatment morphology changes were detected; Fig. 1 in ESIF shows partial nuclei swelling and necrosis characterised by fracture. Live/Dead assay showed similar results; was confirmed drop in cell number at 100 nM concentration, as well
increase in viable/necrotic cell ratio towards necrotic cells (see Fig. 2 in ESI†).

3.3 QDs quantification
The FlowJo software was employed to convert fluorescent emission from cells to relative amount of ingested nanoparticles. Gated consistent cell population with narrow distribution profile was plotted in FITC (green) fluorescence channel. In all samples histograms represented normal distribution.

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

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, due to the fact that the system behaves as a normal distribution.

3.4 QDs internalizing
Flow cytometry allows accurate collection of fluorescent signal which are quantized for each cell. Cells were grown in presence of QDs for 12 and 24 hours, respectively. Control cultures didn’t contain any particles. Comparing flow cytometry data to the results of the PicoGreen study, where no alterations in ds-DNA content in samples treated with the same amount of nanoparticles were observed, it can be concluded that there is no detectable uptake in the case of treatment with concentrations 1–10 nM. Drastic changes were observed for samples exposed to 100 nM. At the 12 hour time point, the tested QDs demonstrated higher fluorescence amplification ratio – compared to untreated cells. However, 12 hours later there was not much difference among all probes (Fig. 3).

3.5 Apoptosis or necrosis?
To answer this question monocyes were cultured for 12 or 24 hours with QDs and subsequently submitted to an Annexin V assay. The Annexin V kit was used to distinguish apoptotic versus necrotic cells stained with fixable viability dye according to protocol. Notable alterations were found in probes treated with 100 nM of QDs. Lower concentrations did not induce any differences compared to control. FITC positive subsets were chosen from histogram overlay of untreated control and 100 nM exposed cells (Fig. 4C and D). The selected sub-population was divided into 4 quadrants in Annexin V vs. Viability dye channels. Contribution of viable, necrotic, early and late apoptotic cells to uptake was calculated from the mean value of QD fluorescence spectra (FTTC). Total uptake was performed as integrated value (number of events in each subset multiplied by mean fluorescence). Fig. 4 represents the resulting signal distribution acquired on 12 and 24 hours respectively.

![Graphs showing QD uptake at 12 and 24 hours](image)

Fig. 3 (A and B) Green TGA QDs uptake after 12 or 24 hours of co-culture with RAW264.7 cells. The concentration range is 1–100 nM. Similar to PicoGreen, low concentration (1–10 nM) did not affect the cells. 2.5-folds fluorescence increase is detected at 12 hours acquisition (A), whereas at 24 hours (B) there’s very little difference from control. It can be explained by massive cell death in between 12 and 24 hours and QDs release in cell culture media. (C and D) Uptake histograms obtained from apoptosis/necrosis assay at 12 (C) and 24 (D) hours tests. X-axis is common logarithm of fluorescent intensity in reference green (FTTC) channel. Y-axis is frequency of data distribution. Red line is control (cells did not treated with nanoparticles), blue line is 100 nM treated cells. The overlap area is excluded from uptake count; only cells in area shifted to the right along x-axis are considered in further analysis as containing QDs.
Fig. 4 The contribution of each subset in total observed fluorescence from FITC-positive sub-population after 12 or 24 hours of co-incubation RAW264.7 monocytes with 100 nM green TGA QDs. Legend: necrotic – dead cells followed necrosis pathway; early A – cells in early apoptosis; late A – cells in late apoptosis; viable – live undamaged cells. At 12 hours time point the strongest signal is produced by the cells in late apoptotic stage. At 24 hours signal level is dropped down due to dead cells cleavage and QDs release in the media.

The general tendency is a small number of early apoptotic cells and a low level of fluorescence from necrotic cells regardless time of exposure. Due to size small and a lack of protective coating, green TGA nanoparticles were quickly ingested by the cells with significant accumulation (85% positive events) resulted in strong signal. It caused a significant shift to late apoptosis stage (86% of FITC positive sub-population) observed after 12 hours of co-incubation. Late apoptotic subset appears as the main contributor to detected fluorescent signal.

The effects observed after longer time of cell-culture in presence of QDs are determined by intracellular processes triggered by trapping foreign species. Introduction to high concentration of small QDs inevitably led to the degradation of the stabilizing shell and further particle aggregation. Rapidly ingested green TGA-capped QDs caused massive apoptosis and, consequently, cell cleavage. A small percentage of surviving cells showed the less uptake and harvested signal from all QD-treated cells studied here. These observations were confirmed by transmission electron microscopy (TEM) microscopy – significant morphological changes (cytoplasm granulation, chromatin condensation, mitochondria blebbing, presence of QDs in nucleus) were spotted for 100 nM treated probes (see Fig. 3 ESI†). Live/Dead assay demonstrated sudden drop in cell number.

3.6 CD80/86 pro-inflammatory markers expression

Foreign bodies ingested by macrophages can cause inflammatory response as defence reaction. CD80/86 are early pro-inflammatory receptors expressed on RAW264.7 cell surface. These two markers were chosen as convenient indicators to monitor the inflammation process triggered by the QDs. CD80/86 expression was measured simultaneously with particle uptake using flow cytometry. Fig. 5 demonstrates the results of the assays. CD86 expression was acquired after 12 hours of cell treatment with nanoparticles, as this marker is activated earlier than CD80 which was measured at the 24 hour time point. Cells co-incubated with low concentrations of QDs (1–10 nM) demonstrated increased levels of both receptors. It was expected that at 100 nM QD concentration, the level of inflammatory markers would be significantly higher due to intensive internalizing and consequent activation of defence mechanism cascades. However the analysed markers were inhibited in this experiment. Fig. 5 depicts observed macrophage behaviour. To investigate on such unexpected effect, cells were activated by PMA followed by QD exposure, the same pattern was detected –

Fig. 5 (A and B) CD80/86 expression of unconditioned monocytes at 12 and 24 hours time-points, respectively. Drastic down-regulation of both markers is observed for cells treated with 100 nM QDs. It’s related to high number of non-functional (necrotic, late apoptotic) cells and hence their failure to proper expression of the surface molecules. (C) CD86 expression after preliminary activation of monocytes with PMA for 6 hours. Cells were exposed to green TGA QDs for 12 hours after priming. The activation is confirmed by elevated production of CD86 comparing to unconditioned cells. The same behaviour is observed in non-primed monocytes, where the CD86 expression is knocked down in 100 nM case. (D) Uptake pattern for the cells activated by PMA for 6 hours and treated with green TGA QDs for 12 hours.
CD86 was suppressed after 12 hours of treatment with nanoparticles.

4. Discussion

Exposure to low doses of QDs (1 and 10 nM) doesn’t affect cell function and viability at any time point. Nanoparticles uptake is not linear process, the saturation level has to be achieved to trigger effective ingestion. Highest tested concentration 100 nM had resulted in massive uptake by the cell and number of consequent effects. Fig. 4 shows total intensity of recorded fluorescence and contribution of each cellular subset. It suggests that green TGA QDs due their small size quickly penetrate cells and cause cell damage and death, what we observe after 12 hours of co-incubation. Afterwards, found impaired cells with high amount of QDs are destroyed physically and release nanoparticles back to the media.

The majority of cells take up QDs in first 2 hours (according to Chitrani and Chan’s model), followed by their cycle shut down and apoptosis trigger. Early apoptosis is observable after further 2-4 hours of co-culture; as it’s quick stage, has not been detected at final 12 hours flow cytometry experiment. Next 4-6 hours late apoptosis is developing, what was observed in experiment. In summary, after 12 hours we have 2 subsets: the small one is without QDs, and the majority one where cells are appeared to ingest nanoparticles, which caused disruption of cell cycle and promoted apoptosis up to late stage. A small amount of live and necrotic cells were also found (1-2%).

In next 12 hours late apoptotic cells are getting eliminating from the system; those survived 1% might undergo 1 division; it gives us a small increase in fluorescent response. We presume that nothing is happening in resistant subset, so it remains neutral to QDs. As result we observe that only 19% of cells have QDs; nearly 60% of this subset are viable and 40% are necrotic. The increase in uptake signal is negligible comparing to 12 hours response.

It has been shown that nanoparticles uptake depends on number of factors, such as particle size, coating, composition, surface charge, shape, protein corona formation, cytotoxicity, cell type. In several works was developed and proved model which states that uptake is happening regardless phase of cell cycle, saturation is achieved once cell underwent full cycle. This study was conducted for non-toxic polymer particles (diameter is ~40 nm). It can be extrapolated to our case, but with certain limitations, as QDs are potent to arrest cell cycle. Besides the doubling time, as well as cell type should be take in consideration: macrophages are professional phagocytes which supposed to ingest and destroy foreign body once it’s recognised as “non-self”. RAW264.7 macrophage-like cell line has been shown as fastest ingesting cell type with high uptake rate. Another feature of this cell line is short doubling time ~ only 11 hours, comparing to HeLa, A549 or U937 cell lines which have 24 hours cell cycle duration.

The prevalent mechanism of nanotoxicity is still under debate. Oxidative stress occurs when cells are treated with nanoparticles and changes mitochondria membrane potential in response. In a classical apoptotic pathway, increased mitochondrial permeability results in cytochrome c release and consequent caspase – 9, 3, 6 and 7 cascades activation. The first target is damaged mitochondria itself and ROS generation. Wilhelmi had showed that this mechanism takes place in RAW264.7 cells treated with ZnO nanoparticles. At the same time the results of TEM analysis suggest the heterogeneity of cell death: necrotic “ghost cells” were also been found as well as apoptotic hallmarkis. Moreover, caspase-independent apoptotic route was shown in caspase-9 deficient Jurkat T lymphocytes. The observed cell death mechanisms “combo” has not been related to any particular factors. Same effect – simultaneous presence of apoptosis and necrosis – was studied by M. Liu in A549 lung cancer cells exposed to 10 nm gold nanoparticles. The presence of caspase-independent apoptosis has also been proved by activity of AIF and EndoG proapoptotic factors – triggers of chromatin condensation and DNA shredding. Interestingly, the experiments had different time scale: 6 hours for monocytes and 72 hours for cancer cells, but the same outcome. That’s in line with the intrinsic cell lines properties – cancer cells are more inert to nanoparticles rather than actively ingesting macrophages. Controversially, Pan et al. observing same pattern in HeLa cells treated with 1.4 nm Au nanoparticles, had excluded apoptosis by the fact that zVAD-fmk inhibitor did not prevent cell death hence only necrosis is happening, regardless fact of massive oxidative stress and mitochondria disruption. Caspase 3/7 activity was tested and did not show significant up-regulation in nanoparticle treated cells, but this is the only apoptosis marker has been examined. Basing on later observations of other groups mentioned here we can speculate that caspase-independent mechanism might have place. Surprisingly, larger (over 60 nm) “non-toxic” silver nanoparticles had continued the trend in causing cell death through both mechanisms. Foldbjerg et al. used THP-1 human leukemia monocyte cell line exposed up to 24 hours to Ag nano-crystals and described “typical” picture – high ROS production, fragmented DNA, large amounts of apoptotic and necrotic cells (AnnexinV/PI assay). It’s hard to say whether coexistence of apoptosis and necrosis has competitive or cooperative nature. Taken together, our results are in concordance with described above cases, unless QDs are considered as potentially highly toxic agents due to presence of Cd and Te and their small size (~2 nm), whereas other studies are dealing with relatively “cell friendly” compounds (Ag, Au, ZnO) and species of similar or greater dimensions. It has to be admitted, that further tests are required to fulfill the knowledge in molecular mechanisms regulating and defying cell fate (apoptosis, necrosis, surveillance) upon the exposure to any nanoparticles, especially to those in a 1-10 nm size range.

It was expected that activity of pro-inflammatory markers CD80/86 will be elevated within the introduced QD concentration. In fact, monocytes did not respond on 1 and 10 nM and got significantly down-regulated when treated with 100 nM. Similar results were observed for other nanoparticles as well, but the source of the phenomenon was not investigated. Tsai et al., 2012 (ref. 53) attempted to explain inhibition of TLR9 signalling by 4 nm gold NPs in either bone marrow derived primary macrophages and RAW264.7 cell line. They attributed this
down-regulation to particles with the largest surface-to-volume ratio for NPs ranged up to 45 nm. Hoshiba et al., 2009 [ref. 54] showed in in vivo and in vitro experiments that CdSe QDs didn’t cause an elevation of anti-bacterial defenders IL-6 and TNF-alpha in peritoneal macrophages, but arrested proliferation of CD4+ T-lymphocytes. It could be related to molecules irresponsiveness to such stimuli as QDs. Thus we carried out another experiment where monocytes were pre-activated by PMA for 6 hours and then treated with QDs for 12 hours. Compared to unconditioned cells, PMA activation had nearly 3 times greater CD86 expression level. The response to low dosage is negligible, whereas 100 nM again arrested CD86 production. We can conclude that the observed dysfunction in both cases is consequence of cell damage caused by ingestion of the QDs at 100 nM concentration. Intriguingly uptake pattern for activated monocytes incubated for 12 hours with QDs is the same as for unprimed cells exposed to the same conditions for 24 hours. This is the result that alerted by PMA monocytes are actively ingesting QDs and accelerate cell damage processes. Thus activated monocytes can be used as active cargo to deliver nanoparticles to target cell or inflamed sites.

5. Conclusion

Flow cytometry was explored for quantification of intracellular QDs. Three different concentrations of QDs (1, 10 and 100 nM) were introduced to cell cultures. Only the highest one - 100 nM - was found effective with regards to uptake. We propose that lower concentrations were unable to form vesicles suitable for ingestion, as it was shown by Chithrani and Chan, 2007. Due to complexity of interaction between QDs and cell culture proteins, surface receptors and cellular organelles, the estimated number of nanoparticles we added to cells is not the same as that detected after certain time of co-incubation. In other words, the initial particles and QDs inside the cells are different species. The advantage of flow cytometry is that it offers a quick measurement of the fluorescent signal from a large number of cells which in turn provides a comprehensive outlook on population level. It helps to evaluate amount of particles taken up without bias. This is important as in final distribution one can find some cells either with low and high fluorescence, whereas geometric mean value is a more accurate representation of population.

The exposure to nanoparticles caused unexpected immune responses: we believed that the expression of pro-inflammatory surface markers (CD86/86) would be upregulated in dose-dependent manner. In fact neither 1 nor 10 nM QDs affected the aforementioned parameters. In the case of 100 nM concentration, both receptors were drastically reduced (less than 50% of control). Given the Annexin V assay results this change is not controversial as the majority of cells with high amount of ingested QDs are apoptotic.

The obtained results address few questions to future investigation. First of all, to evaluate the accuracy of flow cytometry, mass spectrometry should be carried out to make a clear correlation between intracellular cadmium content and observed fluorescence. It will also help to understand how QD fluorescent signal changes after interaction with cellular compartments. Further, it’s always an open question how much cell line results can be extrapolated on primary cells; next step will be measurement same parameters in primary cultures, particularly antigen presenting cells (e.g. monocytes/macrhapsages, dendritic cells). It should include an investigation on mechanism behind cell activation and signalling molecules expression upon QD uptake and exposure.

Acknowledgements

This work was conducted under the framework of INSPIRE, the Irish Government’s Programme for Research in Third Level Institutions Cycle 5, National Development Plan 2007–2013 with the assistance of the European Regional Development Fund and the Ministry of Education and Science of the Russian Federation (Grant no. 14.4253.11.0002). Authors are grateful to Shirley Hanley (PhD, NCBES) for help with flow cytometry experiments and Pierce Lalor (Centre for Microscopy and Imaging, Anatomy Department, NUI Galway) for support with TEM processing and imaging.

References


An experimental and theoretical assessment of quantum dot cytotoxicity†

Olga Gladkovskaya,a,b Paul Greaney,c Yuri K. Gun’ko,d Gerard M. O’Connor,a Martin Meerec and Yury Rochev†b,e

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 recognizes 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 Cd2+ 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 blastocysts and, more recently, primates. 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.

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. While cytotoxicity 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. 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. 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. Models of this type have been extended to incorporate flow cytometry data and have also been specialized for particular types of nanoparticles, such as silicon quantum dots. It has also been found that the process of cellular uptake of nanoparticles can be dependent on the cell cycle phase. Aberg et al. 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.

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 ESI (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 ESI (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
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

$$\frac{dc(t)}{dt} = 0$$

where $k_{s}$ 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

$$c(t) = c_{s} \left(1 - e^{-k_{s}t}\right)$$

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

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. and Wilhelm et al. 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. Fig. 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

$$\frac{dA(t)}{dt} = \frac{1}{4} k_{d} \frac{dA(t)}{dt} + \frac{1}{4} k_{d} A(t)$$

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 $k_{d}(c)$ and $k_{d}$ are rate parameters and $k_{d}(c)$ is concentration dependent. It is natural here to take $k_{d}(c)$ 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 $k_{a}$ to be independent of $c$.

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

$$\frac{dN(t)}{dt} = \frac{1}{4} k_{d} \frac{dA(t)}{dt} + \frac{1}{4} k_{d} N(t)$$

$$\frac{dD(t)}{dt} = \frac{1}{4} k_{d} \frac{dA(t)}{dt} + \frac{1}{4} k_{d} D(t)$$

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

$$k_{a}(c) = \frac{1}{4} k_{a} k_{a}(c)$$

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

$$\frac{dA(t)}{dt} = \frac{1}{4} k_{d} \frac{dA(t)}{dt} + \frac{1}{4} k_{d} A(t)$$

$$\frac{dN(t)}{dt} = \frac{1}{4} k_{d} \frac{dA(t)}{dt} + \frac{1}{4} k_{d} N(t)$$

$$\frac{dD(t)}{dt} = \frac{1}{4} k_{d} \frac{dA(t)}{dt} + \frac{1}{4} k_{d} D(t)$$

We solve the governing equations subject to the initial conditions

$$A(0) = 0, N(0) = 0, D(0) = 0, H(0) = 0$$

This journal is © The Royal Society of Chemistry 2015

Toxicol. Res. 2015, 4, 1409–1415 | 1411
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 eqn (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, eqn (9) implies that $H(t)$ can be solved for independently of $\Lambda(t)$, $N(t)$, 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 eqn (7) and (8), which may now be solved for $\Lambda(t)$ and $N(t)$, respectively. With $\Lambda(t)$ and $N(t)$ available, eqn (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 $k_{i1}$ and $k_{i2}$ since the $c_s$ term only appears in the model equations when combined with one of $k_{i1}$ or $k_{i2}$. 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 modeling 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 Hoosie & Levese, 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 Hoosie et al.22 The software estimated the parameter values using the available experimental dataset.

### 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)

<table>
<thead>
<tr>
<th>Type (units)</th>
<th>$k_e$ (h$^{-1}$)</th>
<th>$k_{i1}$ (h$^{-1}$)</th>
<th>$c_{s1}$ (h$^{-1}$)</th>
<th>$c_{s2}$ (h$^{-1}$)</th>
<th>$k_{i2}$ (h$^{-1}$)</th>
<th>$k_{d2}$ (h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green gel</td>
<td>1</td>
<td>0.05</td>
<td>0.009</td>
<td>0.11</td>
<td>0.05</td>
<td>0.86</td>
</tr>
<tr>
<td>Green TGA</td>
<td>0.81</td>
<td>0.12</td>
<td>0.3</td>
<td>0.06</td>
<td>0.14</td>
<td>0.1</td>
</tr>
<tr>
<td>Red gel</td>
<td>1</td>
<td>0.05</td>
<td>0.148</td>
<td>0.07</td>
<td>0.04</td>
<td>0.48</td>
</tr>
<tr>
<td>Red TGA</td>
<td>0.167</td>
<td>0.05</td>
<td>0.144</td>
<td>0.1</td>
<td>0.04</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Model results

The numerical results are displayed in Fig. 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 Fig. 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.

### 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 ($t = 0\ h, 12\ h, 24\ h$), 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.

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⁻¹ of penicillin and 100 μg mL⁻¹ of streptomycin. Macrophages were maintained in a humidified atmosphere with 5% CO₂ at 37°C.

Annexin V apoptosis assay. In this assay, cells were seeded to a density of 2.5 × 10⁵ 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, resuspended 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 the Mathematical modelling section, 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.

Conflict of interest

None declared.

Acknowledgements

This work was conducted under the framework of INSPIRE, the Irish Government’s Programme for Research in Third Level Institutions Cycle 5, National Development Plan 2007–2013. Paul Greaney is supported by the College of Science, National University of Ireland, Galway. The authors are grateful to Pierce Lalor (Anatomy Department, NUI Galway) for support with TEM processing and imaging.

References

8 H. Jin, D. A. Heller, R. Sharma and M. S. Strano, Size-dependent cellular uptake and expulsion of single-walled carbon nanotubes: Single particle tracking and a generic
In vitro study on the response of RAW264.7 and MS-5 fibroblast cells on laser-induced periodic surface structures for stainless steel alloys†

Clare McDaniel,a Olga Gladikovskaya,a,b Aiden Flanagan,c Yury Rochevb and Gerard M. O’Connora

The manner in which cells interact with a surface is mainly determined by the surface chemistry, surface charge, curvature and micro-topography of the surface. This study investigates the bio response of two metallic alloy coronary stents with Laser Induced Periodic Surface Structures (LIPSS) produced by femtosecond laser pulses at 515 nm and 1030 nm wavelengths. The study relates the bio response to changes in micro-topography and chemical composition. LIPSS were generated in this study by applying femtosecond pulses with a 500 fs pulse duration at a high repetition rate to smooth polished Platinum Stainless Steel (Pt:SS) and 316LSS stent surfaces, with an original roughness value of 2.9 ± 0.2 nm and 1.5 ± 0.2 nm respectively. LIPSS structures were formed by exposure to laser radiation slightly above the applied threshold fluence using a Gaussian laser beam in air. Experiments were performed at two different wavelengths, 1030 nm and 515 nm, to generate different periodic topographies. When the laser wavelength is increased the LIPSS period and depth also increases, thereby increasing the roughness. LIPSS features were characterized using techniques such as Atomic Force Microscopy (AFM) and X-ray Photoelectron Spectroscopy (XPS). This study identifies how LIPSS impact the attachment of monocyte cells (RAW 264.7) and fibroblast cells (MS-5) in vitro. The cellular reactions of un-textured to LIPSS surfaces were compared. It was found using Scanning Electron Microscopy (SEM) that different cells either attached or detached to LIPSS roughness (ranging from 29 nm to 50 nm). Fibroblast cells did not adhere to un-textured surfaces but formed a monolayer on LIPSS surfaces. This indicates that the LIPSS surface is non-toxic. Monocytes show a high affinity to bare un-textured surfaces and failed to firmly attach onto textured surfaces. In the case of stents, it is an advantage that the concentration of monocytes decreases when LIPSS are introduced as this can reduce thrombosis occurring. In the future, laser structured surfaces with various topographies can offer new bio-functionalities in the area of medical implants.

1. Introduction

Cardiovascular disease is a generic name for the family of disorders related to blood vessels. They can lead to heart problems such as heart attack or stroke. Atherosclerosis causes narrowing or blockage of the blood vessel which results in deterioration of cardio-system functions. Implanting a stent can treat the stenosed artery. A stent is a small wire mesh tube that acts as a scaffold used to mechanically hold open the blood vessel. In recent years, drug-eluting stents (DES) have been developed to decrease restenosis. LIPSS could be beneficial to DES. Incorporating a complex surface topography could encourage endothelialisation and limit thrombotic events.

Lasers have been chosen in this study to generate LIPSS and using these structures the cell response can be evaluated. A number of processes can be performed with lasers: cutting, welding, drilling ablation, melting and marking. Lasers offer excellent precision and repeatability, high speed and quality, accuracy, cost efficiency, minimal thermal input and non-contact. Laser processing has gained significant credibility in the medical device industry and is an integral part of the manufacturing, for example, cutting surgical tools, welding endoscopes or pacemaker housings, drilling holes in hypodermic needles and verification laser marking. Of particular relevance to this study is the application of lasers to profile stents. Femtosecond lasers are ideal for surface structuring because they have a minimal heat affected zone, there is rapid heating and cooling and no laser plasma interaction. Laser
Induced Periodic Surface Structures, otherwise known as "ripples", have been fabricated on metal,\textsuperscript{11} polymer,\textsuperscript{12} semiconductor,\textsuperscript{9} and dielectric\textsuperscript{3} surfaces and have been significantly studied since the 1960s. The formation of LIPSS depends on several factors, such as: wavelength, laser fluence, repetition rate, the number of pulses and the angle of the incident laser beam. With metals, LIPSS normally have a period close to the wavelength of the incident beam and are orientated perpendicular to the direction of the polarization of the beam. It has been recently found that materials such as semiconductors, dielectrics and metals achieve LIPSS with a period much smaller than the laser wavelength.\textsuperscript{9} The spatial period of the LIPSS is dependent on the angle of incidence, wavelength and polarization of the laser beam.\textsuperscript{3} LIPSS have a number of applications such as increasing the area and surface energy, altering the hydrophilic or hydrophobic performance of a materials surface,\textsuperscript{13} improving coating adhesion, optics, reducing reflectivity,\textsuperscript{15} and tribology.\textsuperscript{5,11} The chemical composition and topography of a surface has the greatest effect on protein adsorption.\textsuperscript{15} By increasing the surface area there is expected to be an increase in protein adsorption. The mechanisms responsible for ripple formation are still under debate today. There are a number of theories that attempt to explain the formation of LIPSS. Examples of these include excitation of surface plasmon polaritons (SPPs),\textsuperscript{16} self-organization,\textsuperscript{15} the interference between the laser beam and the scattered wave from the surface material\textsuperscript{1} and the influence of surface tension.\textsuperscript{16,17} Advantages of using LIPSS in this study is that the surface area of a material can be increased in air quickly without the aid of chemicals and stents can be fabricated with various topographies depending on the desired application.

In vitro bio systems were used to model the response of immune cells that circulate in the blood stream of a blood vessel. RAW264.7 cells represent peripheral blood circulating monocytes. Their primary function is monitoring and first line defense against foreign bodies. MS-5 fibroblasts originate from connective tissue and are used in this study to model the response of a blood vessel exposed to a stent.

Monocyte and fibroblast cell types are used in this study to investigate the biocompatibility of a biomaterial, in this case clinically approved stent material. Monocyte adhesion and infiltration occurs instantaneously after injury to the artery. RAW264.7 is a monocyte cell line which fully possesses immune responses including activation into macrophages, expression pro-inflammatory surface markers, inflammatory proteins and stimuli for T-cell recruitment. RAW264.7 murine monocyte/ macrophage cells have been used in a number of in vitro biomaterial studies.\textsuperscript{18-20} RAW264.7 offer diversity in a number of applications: monocytes can be converted to osteoclasts in the presence of receptors for nuclear factor kappa B ligand (RANKL).\textsuperscript{21} In addition they are easy to transfact and activate into mature macrophage phenotype. Alternatively, RAW264.7 cells can be used as naïve monocytes. They have been used to describe biomaterial properties, such as capacity to trigger inflammatory response\textsuperscript{22} or suppress macrophages.\textsuperscript{23} Attract circulating monocytes, ingestion by immune cells, facilitate cell adhesion and growth as immune cells or osteoclasts. It has been found that RAW264.7 cells display a higher rate of adhesion on smooth surfaces compared to rougher surface where the cells begin to differentiate.\textsuperscript{24} Other studies that investigated the performance of RAW264.7 cells on smooth and rough surfaces did not give a definitive answer: some groups suggested that monocytes have an affinity to smooth surfaces,\textsuperscript{25,26} while others reported no difference,\textsuperscript{27} or others observed monocyte activation on flat samples.\textsuperscript{28} Lee et al.\textsuperscript{29} found a higher concentration of monocyte cells present on the surface of textured titanium, with a roughness value of 4.76 nm, compared to un-textured samples, with a roughness value of 0.37 nm.

Fibroblast cells are connective tissue cells that play a crucial role in wound healing. MS-5 murine fibroblast cells do not have direct applications in in vitro studies, but they have positive effects on vasculogenesis and haemopoiesis.\textsuperscript{30} Fibroblast cells are one of the most common cells used in in vitro biomaterial testing and have been used to investigate stent surfaces in previous studies.\textsuperscript{31,32} Also they promote growth of primitive haematopoietic progenitor CD34 + human cord blood cells\textsuperscript{33} and differentiation of murine embryonic stem cells.\textsuperscript{34} It has been found that fibroblast cells adhered more to smooth samples compared to laser treated 316LSS samples.\textsuperscript{35} On silicon, it was found that fibroblast growth decreases with increasing surface roughness (smooth 88 nm, rough from 378 nm to 650 nm).\textsuperscript{34} Pennisi et al.\textsuperscript{36} found that fibroblast cells are more elongated and their cytoskeleton is less mature on rough platinum surfaces (11 nm) compared to smooth surfaces (0.65 nm). The growth rate decreases as the surface roughness increases (11 nm to 23 nm). Li et al.\textsuperscript{37} found that after laser texturing of 316LSS there was a reduction in cytotoxicity for endothelial cells grown on laser-textured surfaces compared to un-textured surfaces. They also found using XPS that the surface chemistry changes with 316LSS LIPSS. There was a reduction in the concentration of iron and nickel with an increase in chromium. To our knowledge the cellular interaction with LIPSS structures of different topographies on metal alloys has not been investigated to date.

2. Materials & methods

2.1. Materials

This study focuses on two alloys; 316L Stainless Steel (SS) and Pt:SS. Pt:SS is composed of 316L stainless steel with the addition of platinum. The overall composition of Pt:SS comprises of 33% platinum, 37% iron, 18% chromium, 9% nickel, 3% molybdenum and traces of manganese. Platinum is used because it creates stronger and more flexible stent struts. Platinum based stainless steel alloys are useful due to their resistance to corrosion and high melting temperatures. 316LSS is a chromium-nickel-molybdenum alloy consisting of 61-72% iron, 16-18% chromium, 10-14% nickel, 2-3% molybdenum, 2% manganese, 0.08% carbon, 0.7% silicon, 0.45% phosphorus, 0.03% sulphur and 0.1% nitrogen. 316L stainless steel is known for its strength, biocompatibility and it is relatively easy to machine due to the carbon present. Metallic stents are preferred because of their radiopacity properties. The Pt:SS and 316LSS stents used in this study have been electro-polished to
create smooth R1 surfaces with an average roughness value of 2.9 ± 0.2 nm and 1.5 ± 0.2 nm respectively. The curvature of the Pt:SS and 316LSS struts are calculated as 9.02 ± 0.64 mm⁻¹ and 8.59 ± 0.22 mm⁻¹, respectively. The characteristics of a stent are different to a flat sample. Studies performed on stent materials, in vivo, have limitations due to the stent size, pattern and curvature and the challenge to analyse the contact angle, surface energy and cell behaviour.

2.2. Laser processing
Pt:SS and 316LSS surfaces were exposed to multiple incident laser shots in air at a repetition rate of 100 kHz at various pulse energies ranging from 2 μJ to 6 μJ. Experiments were carried out using a Yb:KYW chirped-pulse-regenerative amplification laser system (Amplitude Systemes S-pulse HP) that delivered laser pulses with a duration of approximately 500 fs at wavelengths of 1030 nm and 515 nm. The spatial profile of the laser was Gaussian in nature with a nominal M² value of <1.2. The number of pulses is controlled using a computer controlled fast electro-optical modulator (EOM). The pulse energy was controlled using a rotary half-wave plate and a beam splitting polarizing cube.

The process of fully treated stents with LiPSS is shown in Fig. 1. The stent is placed on a 0.051" mandrel. This mandrel is held at one side by a rotary stage and the other side by a collet. A rectangular array consisting of 45 000 laser spots was fabricated on the stent slightly above the threshold fluence. Each spot was exposed to 30 successive laser pulses. The stent was then rotated using a motion system. This procedure was repeated until the stent was fully treated with LiPSS.

The calculations for determining applied threshold fluence (Φth) and Φth are obtained according to the method of Liu et al.17 The spatial fluence, Φ(r), for a Gaussian beam is given by:

\[ \Phi(r) = \phi_0 e^{-2r^2/\alpha_0^2} \]  

where \( \phi_0 \) is the peak fluence in the beam, \( r \) is the distance from the centre of the beam and \( \alpha_0 \) is the Gaussian spot radius (1/e²).

The maximum fluence and the pulse energy, \( \Phi_{th} \), are related by:

\[ \Phi_{th} = \frac{2F_{th}}{\pi \alpha_0^2} \]  

The peak fluence is related to the diameter of the ablated spot.

\[ D^2 = 2\alpha_0^2 \ln \left( \frac{\phi_0}{\Phi_{th}} \right) \]  

where \( D^2 \) is the maximum diameter of the damaged region zone. It is possible to determine the beam radius using the value for \( \phi_0 \) from the plot of \( D^2 \) versus the logarithm of the pulse energy. Once \( \phi_0 \) is calculated fluence values can then be found using eqn (1). By plotting \( D^2 \) versus the natural log of the applied laser fluence and extrapolating the \( D^2 \) line to zero, \( \phi_0 \) can be calculated.20 The representative diameters of the ablated spots were measured using an optical microscope three times to determine an average value.

2.3. Characterisation
LiPSS are generated slightly above the applied damage threshold fluence and then analysed using a SEM (Hitachi S2600N). The topography of the LiPSS was analysed using an AFM (Agilent 5500). The scan was performed using a contact mode tip (Nanosensors TM PPP-CONTR-10, resonance frequency 6–21 kHz, tip height 10–15 μm, force constant 0.02–0.77 N m⁻¹). A 10 μm² area was performed at a speed of 0.5 lines per second and a resolution of 1024. The surface roughness was calculated using PicoImage Advanced (Agilent Technologies) software. Chemical analysis was performed using XPS (Kratos Axis 165) to see if there is a change in the elemental composition with increasing laser pulses. Samples were exposed to 15 seconds of Ar cluster sputtering to decrease the carbon content present on the surface. The area of analysis was 110 μm and selected at random. The XPS values are highly dependent on the location where the scan was obtained inside the spot.

2.4. Cell adhesion experiments
The key challenge in design was to ensure the mandrels did not move during cell seeding and adhesion. Cells were seeded and incubated on stents placed on mandrels and clipped in place.
with stainless steel clips (Supaclip 40, Rapesco) shown in Fig. 2. The mandrels were positioned on Teflon (1.5 mm thick), which was laser cut to allow no contact of Teflon to the stent, shown in Fig. 2. A Picosecond laser (TrumpTrumicro 5050) was used at a repetition rate of 400 kHz at 1030 nm using 150 passes at 2.5 m s⁻¹. The laser was also used to create grooves on the Teflon to position and fix the mandrels in place. The Teflon holder allowed the stent to be as close to the bottom of the petri dish as possible. The clips kept the stent fixed in place to ensure no movement of the stent whereby cells could detach. A sufficient volume of cell culture media was used to ensure the stents were fully covered.

RAW264.7 monocyte-macrophage and MS-5 fibroblast murine cell lines were used in this study. It is important to note that human pulmonary microvascular endothelial cells (HPMEC) were also investigated and showed a positive response to flat surfaces but as there was no difference between bare and rippled stents these results are not included in the current study. The images are available in the ESI† Fig. A. Cells were grown in Dulbecco’s Modified Eagle Media (DME; Sigma), supplemented with 10% Foetal Bovine Serum (FBS; Sigma), 100 µg mL⁻¹ of penicillin and 100 µg mL⁻¹ of streptomycin. Cells were maintained in humidified atmosphere with 5% CO₂ at 37 °C. On the day of the experiment, confluent cell flasks were removed from the incubator. Cells were harvested using enzyme for fibroblasts or fluid wash for monocytes according to routine procedure. Cells were spun down at 1500 rpm for 5 minutes and the final cell pellet was re-suspended in fresh culture media. Cells were seeded onto the stent and left to attach for 6 hours in the incubator. Afterwards, the reduced media was removed and fresh aliquot was added. The next day, the samples were fixed with 2.5% glutaraldehyde and 2.5% paraformaldehyde in phosphate buffered saline (PBS) overnight at room temperature. The cells were then dehydrated in ascending dilutions of ethanol and critically point dried (Quorum Technologies K850 CPD). Samples were gold coated using a sputter coater (EMSCOPE SC500) and viewed under the SEM.

2.5. Live/dead assay

Cell viability was tested using a live/dead assay (Live Technologies, Bio-Sciences, Dun Laoghaire, Ireland) according to the manufacturer protocol. Titration was performed for both calcin and ethidium bromide to define optimal dye concentration. RAW264.7 cells were seeded onto flat SS, PtSS stent and 316LSS stent samples in high 1 x 10⁶ cells per well concentrations. After 24 hours of co-culture, cell viability was examined. A VarioSkand Flash plate reader was used to harvest a fluorescent signal from flat samples.

3. Experimental results

3.1. LISSS

Each stent was fully textured by combining an aligned array of spots at a laser wavelength of 515 nm and 1030 nm. Each spot was exposed to 30 successive laser pulses. The spot radius and threshold fluence were calculated using eqn (2) and (3). The applied threshold fluence of PtSS for 1 pulse was previously calculated and it was found that with increasing number of laser pulses, the threshold fluence decreases. The estimated applied threshold fluence of PtSS for 30 pulses at 1030 nm and 515 nm, are \( \phi_{th} = 0.12 \pm 0.02 \) J cm⁻² and 0.10 ± 0.01 J cm⁻² respectively. The applied threshold fluence of 316LSS for 30 pulses at 1030 nm and 515 nm, are \( \phi_{th} = 0.11 \pm 0.02 \) J cm⁻² and 0.06 ± 0.01 J cm⁻² respectively. There is a decrease in threshold fluence with decreasing laser wavelength. PtSS and 316LSS LIPSS were generated slightly above the applied threshold fluence at \( \phi = 0.2 \) J cm⁻² with 30 pulses for 515 nm and 1030 nm.

3.2. AFM

The topography of PtSS and 316LSS LIPSS on stents were analysed using AFM. Un-textured surfaces were compared against 515 nm and 1030 nm laser wavelength LIPSS. Changing the laser wavelength from IR to visible led to different LIPSS features such as various period and depth sizes thereby changing the roughness. The 3D and side profiles of each parameter are shown in Fig. 3. The grain structure of 316LSS is clearly-visible in Fig. 3(d) with grains approximately 10–15 µm in size. From Table 1, there is an increase in the LIPSS period and depth with increasing wavelength. In the case of PtSS the period has doubled, with an increase in depth of 105 nm, from 515 nm to 1030 nm. For 316LSS, the period has doubled, with an increase in depth of 64 nm, from 515 nm to 1030 nm. The largest estimated increase in surface area is 32% associated with PtSS 1030 nm LIPSS. There is an increase in the roughness with increasing wavelength. From 515 nm to 1030 nm, the surface roughness of PtSS has increased from 35 ± 3 nm to 50 ± 3 nm and of 316LSS has increased from 29 ± 2 nm to 48 ± 3 nm.

3.3. Live/dead assay

A live/dead assay was performed on un-textured flat SS surfaces, 515 nm LIPSS and 1030 nm LIPSS of RAW 264.7 cells. LIPSS were generated using 0.2 J cm⁻² at 100 kHz. There is a high concentration on an un-textured flat surface, shown in Fig. 4(a). This verifies that the surfaces are non-cytotoxic. There is a decrease in the number of live cells attached to the surface with increasing LIPSS period and depth, shown in Fig. 4(b) and (c). Fig. 4(d) is a magnified image of cells grown on 1030 nm LIPSS illustrating healthy morphologies. This implies that cells respond to a change in surface morphology or chemistry.

There is a decrease in the fluorescence of live cells (calcine) with increasing LIPSS period and depth for 515 nm and 1030 nm, respectively, shown in Fig. 4(a–c). This is verified in the corresponding live/dead assay fluorescent values. The number of dead cells (EthD) is insignificant in all cases. There is a high fluorescent value for bare media indicating that protein and fluorescent dye absorption has occurred on the metal; this suggests that it is not an effective control.

There are limitations with performing a live/dead assay on a stent surface due to the decreased surface area and the low concentration of adhered cells onto the surface. When this assay was performed on stents no signal was detected because the fluorescence was below the detection limit of the apparatus.
Fig. 3 AFM topography of Pt:SS (a) un-textured, (b) 515 nm LIPSS and (c) 1030 nm LIPSS, 316LSS (d) un-textured, (e) 515 nm LIPSS and (f) 1030 nm LIPSS. LIPSS were generated at 100 kHz for 30 pulses at 0.2 J cm\(^{-2}\).

<table>
<thead>
<tr>
<th></th>
<th>Pt:SS</th>
<th>316LSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un-textured</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roughness, (R_n) [nm]</td>
<td>2.9 ± 0.2</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Roughness, (R_{rms}) [nm]</td>
<td>34.5 ± 3.3</td>
<td>49.6 ± 3.0</td>
</tr>
<tr>
<td>Surface area increase (%)</td>
<td>19.2 ± 0.9</td>
<td>32.5 ± 2.0</td>
</tr>
<tr>
<td>Period [nm]</td>
<td>388.7 ± 9.0</td>
<td>740.8 ± 13.9</td>
</tr>
<tr>
<td>Depth (nm)</td>
<td>86.1 ± 1.7</td>
<td>177.0 ± 5.2</td>
</tr>
</tbody>
</table>

3.4. SEM

SEM was used to visualize cell adhesion on the surface of the curved struts. Flat SS samples were used as a control to identify the role of curvature and eliminate surface area issues. Curved and flat samples that were treated with cell culture media only were used as a negative control. There appears to be minimal
protein deposition on the curved control samples, shown in Fig. 5 and 6 for Pt:SS and 316LSS respectively.

As shown in Fig. 5 and 6, when a curved non-textured sample is introduced RAW264.7 monocytes attach onto the surface and grow without activating into macrophages. Following laser exposure, fibronectin threads can be easily seen between the cells, shown in figures (iv(c)). Also, large RAW264.7 clusters were found. In case of fibroblasts, they failed to make a firm connection with the curved surface. With LIPSS covered stents, monocytes appear as single cells, loosely adhered to the surface in figures (iii(b)) and (iii(c)), with some cells undergoing apoptosis. Fibroblasts formed a monolayer with healthy morphologies in the case of 1030 nm LIPSS unlike 515 nm LIPSS.

3.3. Surface chemistry

XPS was used to characterize the change in the elemental concentration of Pt:SS and 316LSS after femtosecond laser exposure. Table 2 illustrates the significant differences in concentrations between a bare un-textured surface, 515 nm LIPSS and 1030 nm LIPSS. The elements in Pt:SS 515 nm LIPSS that have the largest percentage variation compared to un-textured Pt:SS are oxygen, iron and molybdenum. At 1030 nm, the leading percentage variations are oxygen, platinum and iron. The oxide layer has increased by 4.7% with 1030 nm LIPSS. For 316LSS, oxygen, nickel and molybdenum have the largest percentage variations at 515 nm. There is a difference response in iron and nickel between the two alloys. For 1030 nm, iron, chromium and molybdenum have the largest percentage difference. After femtosecond exposure, the surface chemistry for both alloys has changed.

4. Discussion

There is no measured difference in the applied threshold fluence for Pt:SS and 316LSS for 1030 nm based on 30 pulses, yet there is a decrease in \(\phi_{th}\) of 0.10 ± 0.01 J cm\(^{-2}\) for Pt:SS and 0.06 ± 0.01 J cm\(^{-2}\) for 316LSS for 515 nm. A decrease in threshold fluence with decreasing wavelength is found, which implies that there is less energy needed to ablate 316LSS at shorter wavelengths. This reduction can be attributed to changes in elemental composition or reflectivity.
AFM was used to analyse the LIPSS structures formed on Pt:SS and 316LSS. The period of the surface ripples is significantly smaller than the wavelength of the incident laser beam. In the case of Pt:SS, the period is estimated to be 740 ± 14 nm and 389 ± 9 nm when exposed to a 1030 nm and 515 nm laser beam, respectively. It was found that the LIPSS period increases with increasing laser wavelength. For a metal, the period of LIPSS (\(A\)) is related to the wavelength using the equation: \[ A = \frac{\lambda}{1 \pm \sin \theta} \] (4)

where \(\lambda\) is the wavelength of the incident beam, \(\pm\) represents the back and forth propagating surface wave at the surface of the metal and \(\theta\) is the incident angle of the laser on the surface of the material. Using eqn (4), \(A\) is predicted accurately at \(\theta = 25^\circ\) for 316LSS but does not capture the behaviour of Pt:SS.

The depth of the LIPSS also increases with increasing wavelength. The depth of ablation depends on the absorption coefficient, thermal diffusion and radiative cooling of the material. Because \(a\) is very high for metals (\(\approx 10^6 \text{ cm}^{-1}\)) the incident laser energy is absorbed between a 10–20 nm depth.\(^{38}\) The effective penetration depth (\(\alpha^{-1}\)) is proportional to the laser wavelength. For platinum, \(\alpha^{-1}\) is estimated to be 11 nm for 515 nm and 13 nm for 1030 nm. Hence, there is a marginal difference in depths between the incident wavelengths used in this study. Using AFM, for Pt:SS, the depth of LIPSS increased from 86 ± 2 nm to 177 ± 5 nm from a wavelength of 515 nm compared to 1030 nm; for 316LSS the depth has increased from 78 ± 3 nm to 142 ± 5 nm. This increase in depth found experimentally could be due to changes in the material after the laser interaction has ended. After exposure from a femtosecond pulse the alloy material is likely to become amorphous thereby changing its optical penetration depth.\(^{44,45}\) Likewise, after multiple-pulse exposure incorporating rapid heating and cooling cycles, the alloy could evolve towards a metallic-like glass thus transforming the optical properties of the material.\(^{46}\) This would allow for deeper surface structures.

The heat diffusion length, \(l_t\), is calculated by \(2(Dt)^{1/2}\), where \(D\) is the heat diffusivity and \(t\) is the laser pulse duration.\(^{39}\) For
stainless steel $D$ is 0.04 cm$^2$ s$^{-1}$ (ref. 38) and within a laser pulse duration of 500 fs, $\tau_d$ is calculated to be approximately 2 nm. For platinum ($D = 0.25$ cm$^2$ s$^{-1}$), $\tau_d$ is calculated to be 7 nm. The heat diffusion length within a laser pulse is underestimated and does not capture the depths measured using AFM. With multiple-pulse exposure, there could be an accumulation of heat, which increases in depth after each successive pulse thereby increasing the total depth of the LIPSS. Another factor that could contribute to a larger heat depth is the diffusion of ballistic electrons into the bulk of the material. Ballistic electrons are high-energy electrons that travel into the bulk of the material before thermalization.

The largest measured increase in surface area of 32% is associated with Pt:SS 1030 nm LIPSS and is attributed to the larger depth. Another measure of area, the surface roughness, has increased by approximately 29.5 ± 2.2 nm and 46.6 ± 0.2 nm for 515 nm and 1030 nm respectively.

On the biological side, one of the main challenges in this study was the limited number of suitable bioassays. The stents in this study are metallic, have a small diameter and length, an appreciable strut curvature, narrow struts and therefore a small surface area available for cell adherence and growth. Only a small number of cells are available for analysis. Fluorescent microscopy on a non-transparent 3D object is difficult to perform, especially at high magnification. The structure of the stent brings another level of complexity, as cells that are attached on other struts can create a strong fluorescent background, which obstructs focusing on a particular plane during fluorescent analysis. For any quantitative test like a live/dead assay, PicoGreen, Alamar Blue or ELISA, the number of cells per sample that we measured were too low to produce statistically relevant results even when cells demonstrate compatibility with the laser structured surface. Furthermore, in cases when cells failed to adhere it was not possible to detect their presence. Due to these issues, SEM has been chosen as the main technique for characterization of the bio response. It allows us to evaluate sample cytotoxicity and study important parameters such as cell attachment, growth, morphology, activation status.
Table 2  % concentration for Pt:SS and 316LSS elements using XPS after exposure to 30 pulses at a repetition rate of 100 kHz at 0.2 J cm⁻²

<table>
<thead>
<tr>
<th>Element</th>
<th>No laser exposure (bare)</th>
<th>515 nm LiPSS</th>
<th>% variation</th>
<th>1030 nm LiPSS</th>
<th>% variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt</td>
<td>58.24 ± 1.00</td>
<td>62.60 ± 0.69</td>
<td>+6.36</td>
<td>62.91 ± 0.87</td>
<td>+6.67</td>
</tr>
<tr>
<td>Cr</td>
<td>16.17 ± 0.27</td>
<td>17.41 ± 0.16</td>
<td>+1.24</td>
<td>17.65 ± 0.28</td>
<td>+1.24</td>
</tr>
<tr>
<td>Ni</td>
<td>13.44 ± 0.63</td>
<td>13.60 ± 0.49</td>
<td>+0.16</td>
<td>13.95 ± 0.51</td>
<td>+0.49</td>
</tr>
<tr>
<td>Fe</td>
<td>1.67 ± 0.64</td>
<td>0.97 ± 0.43</td>
<td>−0.74</td>
<td>0.14 ± 0.61</td>
<td>−0.33</td>
</tr>
<tr>
<td>Mo</td>
<td>8.28 ± 0.63</td>
<td>5.43 ± 0.45</td>
<td>−2.85</td>
<td>9.89 ± 0.65</td>
<td>+1.61</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>316LSS [% Concentration]</th>
<th>No laser exposure (bare)</th>
<th>515 nm LiPSS</th>
<th>% variation</th>
<th>1030 nm LiPSS</th>
<th>% variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>70.36 ± 1.00</td>
<td>74.59 ± 1.03</td>
<td>+4.63</td>
<td>70.29 ± 0.90</td>
<td>−0.27</td>
</tr>
<tr>
<td>Cr</td>
<td>16.79 ± 0.61</td>
<td>16.52 ± 0.46</td>
<td>−0.27</td>
<td>14.80 ± 0.51</td>
<td>−1.99</td>
</tr>
<tr>
<td>Ni</td>
<td>1.96 ± 0.84</td>
<td>0.24 ± 0.58</td>
<td>−0.62</td>
<td>1.73 ± 0.74</td>
<td>−0.13</td>
</tr>
<tr>
<td>Fe</td>
<td>8.27 ± 0.69</td>
<td>8.07 ± 0.62</td>
<td>−0.23</td>
<td>11.27 ± 0.79</td>
<td>+3.09</td>
</tr>
<tr>
<td>Mo</td>
<td>2.52 ± 0.49</td>
<td>0.59 ± 0.42</td>
<td>−1.93</td>
<td>1.90 ± 0.43</td>
<td>−0.62</td>
</tr>
</tbody>
</table>

For immune cells, cluster formation, fibronectin webbing and protein deposition. None of the tested samples demonstrated significant cytotoxicity as cells with a healthy morphology are present.

It is intriguing that cells showed notable selectivity to the surface modifications on the structured stent. RAW264.7 monocytes have a clear preference for unstructured metal surfaces. Cells were found with healthy morphologies with few that activated into macrophages. Big cell clusters with dense fibronectin connections were also observed. Only swollen dead cells were found on LiPSS surfaces. Factors that can contribute to the adhesion and proliferation of cells on surfaces are surface topography, chemical composition, surface free energy, stiffness and electrostatic charge density. A change in any of these factors could result in the change in response of RAW 264.7. Using a different cell type, Misra et al. found that osteoblast cells had the largest response to hydrophilicity and grain structure on the surface of stainless steel.

MS-5 cells failed to make a firm connection with unstructured surfaces. In addition these cells were unable to attach onto the 515 nm LiPSS. In the case of LiPSS generated by 1030 nm, the MS-5 cells attached and formed a monolayer with healthy morphologies. The deeper LiPSS could create a “keyhole” effect thereby alloying the MS-5 cells to attack and proliferate. This demonstrates that MS-5 cells prefer a roughness value of approximately 50 nm. The adhesion, growth and spreading of MS-5 cells on the surface of 1030 nm LiPSS verifies that the surface is non-toxic. HPMEC cells were also examined on the surface of bare and textured stents in vitro yet the results were not consistent. More detailed work is needed to identify surface patterns that suit endothelial cells.

The XPS study was performed to detect chemical changes on the alloy surface after exposure to multiple femtosecond pulses at different laser wavelengths. It was also used to interpret the response from the tested cell lines. From the XPS measurements, it is clear there is a redistribution of elements at the surface after laser exposure. XPS can penetrate the surface to a depth of approximately 10 nm therefore XPS examines the oxide layer and bulk material. Table 2 shows changes to the following elements. The oxide thickness of a bare Pt:SS stent surface is estimated to be 1.5 nm. The oxide thickness of 316LSS was calculated to be 3.6 nm. There is an increase in oxide thickness with an increasing wavelength for Pt:SS. The contrary is observed for 316LSS, there is a decrease in oxide thickness from 515 nm to 1030 nm. For Pt:SS, there is a 6.3% decrease in Pt at 1030 nm.

It is unlikely that the oxide layer and concentration of Pt is a factor that affects the response of MS-5 and RAW264.7 cells because both alloys have the same cell response. In relation to Cr and Ni, there is a decrease in both elements implying that the surface has become less toxic, except for structures generated on Pt:SS with 515 nm. While this should have a positive impact on the concentration of RAW264.7 cells from bare to 1030 nm the opposite is observed in this study. In the case of bare to 515 nm there is also a decrease in the concentration of Fe. There is an increase in the concentration of Fe from bare to 1030 nm and both alloys have a high percentage of this element present. 37% Fe in Pt:SS and 61%–72% in 316LSS. There is no explicit indication on how cells respond to the Fe content in alloys. Some studies show that the cytotoxicity increases in a Fe based alloy in vitro, which reduces viability of fibroblasts while others show an increase in fibroblast proliferation. Based on the XPS results in this study, it is assumed that fibroblasts are susceptible to Cr and Fe, which corresponds to a decrease in Cr and an increase in Fe. It is as yet unclear what chemical changes drive cell response.

5. Conclusion

Applied threshold fluence values were calculated on Pt:SS and 316LSS at 100 kHz for 30 pulses at 1030 nm and 515 nm. There is a change in the threshold fluence when decreasing
wavelength. There is an increase in the LIPSS period and depth with increasing wavelength. Using two wavelengths achieves different topographies. It was found that the parameter to give the highest surface area was 100 kHz at 1030 nm for 30 pulses on PtSS with a 32% increase. Also, there is an increase in the roughness with increasing wavelength.

Using SEM, it was determined that different cell types react differently to surface roughness's on a curved surface. Monocytes prefer un-textured surfaces with a roughness value of several nm, while MS-5 cells prefer a surface with a roughness value of approximately 50 nm. Similar cell responses were observed on PtSS and 316LSS stent surfaces. On a flat surface it was shown that the number of adhered cells decreases with increasing LIPSS period and depth, confirmed using a live/dead assay. This verifies that cells respond to a change in surface morphology and/or chemistry. This also concludes that with the introduction of LIPSS the surfaces are non-cytotoxic, as healthy morphologies are present. Flat samples were used to prove the biocompatibility of the alloy surface after laser exposure. Because RAW 264.7 and MS-5 cells successfully attached to all un-textured and LIPSS covered surfaces, it is safe to assume that stent topography is a large factor in cell growth. It was found using XPS that the surface chemistry changes from bare to 515 nm and 1030 nm for both PtSS and 316LSS. It is assumed that cells respond to changes in Cr and Fe but there is no difference in cell interaction between the two alloys. From this, it is concluded that surface topography generated by ultra-short lasers greatly influences cell behaviour. The surface chemistry does not change significantly but the topography does. Although this study is performed in vitro one cannot assume the same responses could occur in vivo. A stent could be fabricated with various LIPSS morphologies depending on the application. LIPSS allows one to quickly increase the surface area and roughness of a surface without the use of chemicals. The integration of laser-structured implants with specific topographies may offer new bio-functionalities in the future. The stents used in this study (PtSS and 316LSS) have already been developed and approved for clinical relevance. This study focuses on surface modifications on a stent and recording a bio-response. We propose that this in vitro study is a good starting point from which in vivo studies can progress to clinical trials in the future.

Acknowledgements

This work is supported through an IRCSET research grant no. IRCSET-BS-2011-01 and was conducted under the framework of the INSPIRE programme, funded by the Irish Government’s Programme for Research in Third Level Institutions, Cycle 4 + 5, National Development Plan 2007–2013. Sincere thanks to Pierre Lalor and Dr Kerry Thompson at the Centre for Microscopy and Imaging (CMI) at the Anatomy Department in NUI, Galway for their assistance.

References


Water-based ultrasonic synthesis of SbSI nanoneedles

Olga Gladkovskaya a,b, Irina Rybina c, Yurii K. Gun’ko d,e, Andrea Erxleben f, Gerard M. O’Connor g, Yury Rochev b,f,n

a School of Physics, National University of Ireland, Galway, Ireland
b Network of Excellence for Functional Biomaterials, Galway, Ireland
c School of Chemistry, Analytical Chemistry Department, Southern Federal University, Rostov-on-Don 344090, Russian Federation
d CIANN and School of Chemistry, Trincky College, Dublin, Ireland
e TIFG University, 197101 St Petersburg, Russia
f School of Chemistry, National University of Ireland, Galway, Ireland

Abstract

Semiconducting nanomaterials have found a range of applications in optoelectronics, energy harvesting, sensing, bio-imaging etc. Nanomaterials should be easy and inexpensive to manufacture, with stable and reproducible characteristics over time. Antimony sulpho-iodide (SbSI) has a unique combination of semiconductor, optic and piezoelectric parameters, however, its applications are currently hampered by difficulties in its production. Previous studies and experiments carried out to synthesise SbSI crystals involved long reaction times (over 70 h), and high temperatures (E 250 °C).

In this work we developed water-based synthesis to produce sub-micron sized crystals and explore their properties. We attempted to control SbSI particles growth and formation by adding different surfactants and fillers to the reaction system. The crystal dimensions (shape/size) varied depending on the added filler. In addition, we performed in vitro cytotoxicity studies of SbSI particles and evaluated their potential for biomedical applications.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Antimony sulpho-iodide (SbSI) is widely studied due to diverse properties: semiconducting, ferroelectric, photoconductivity, piezoelectric, pyroelectric and pyro-optic [1–7]. SbSI has the highest Curie temperature (Tc) of any of the V–VI–VII class of ternary materials at approximately 21 °C [1, 5], the highest dielectric constant along the polar axis, and the highest known refractive index (n) of any material of 4.5 along the c-axis [3, 6]. This refractive index adds to the importance of SbSI in the development of electro-optic devices, although this is dependent on large defect-free crystals becoming readily available. SbSI has a band gap of 2.12 eV, which shows abnormally high temperature coefficients [4]. These properties of SbSI make this material very promising for applications in displays, actuators, solar cells, nonvolatile memory devices, piezo- and pyroelectric detectors [1, 8].

Various techniques have been developed to produce and texture SbSI in its different modifications. Flash and beam evaporation methods were first reported for thin film assisted manufacture [9–12]. Hydrothermal synthesis, sonication in ethanol, vapor transport reaction, refluxing were considered by number of groups for SbSI and its derivatives [2, 4, 8, 10, 13–15]. All methods suffer either from long reaction time, high temperature/pressure, low product yield, or small scale synthesis. A simple and elegant water-based synthesis route has been described by I. Rybina in 1998 [16]. It allows production of pure crystal SbSI from easy available precursors in large scale at room temperature. The only downside of this technique is poor control of reaction kinetic and thus crystal size/shape.

The main goal of our work was to develop and optimize the synthesis of SbSI micro- and nanocrystals, investigate their optical properties and in vitro toxicity. We also explored the influence of physical and chemical parameters on the kinetics of synthesis and crystal formation. The reaction is found to be extremely sensitive to pH and solution ionic strength. Treatment with ultrasound helps to improve the final product purity and crystallinity. The crystal shape has been drastically changed upon adding surfactants. The cytotoxicity of as-prepared material has also been tested.

http://dx.doi.org/10.1016/j.matlet.2015.07.027
0025-5408/© 2015 Elsevier B.V. All rights reserved.

113
2. Materials and methods

All chemicals used in the study were purchased from Sigma Aldrich. Antimony sulpho-iodide was synthesized as previously described [17,18]. To modify crystal properties, 1 mM of fillers (Sodium dodecyl sulfate (SDS), L-ascorbic acid, acetic acid, polyvinylpyrrolidone (PVP), thiglycolic acid (TGA), Tween 80, hexadecyltrimethylammonium bromide) and sodium chloride were added. A high power ultrasonic tip was used to break down the particles to nano-scale level.

As-prepared ShS1 was examined for purity and crystal structure by XRD (Icel Equinox 6000). No contaminating residues were found, all typical peaks for Pmaa phase are presented. The obtained precipitates were described by SEM, EDX (Hitachi S-4700) and TEM (Hitachi H7500). The products of high-power ultrasonic treatment were examined for absorbance/fluorescence on Shimadzu UV-1601 and Cary Eclipse spectrometers.

Alamar blue® was used to examine material biocompatibility. RAW264.7 murine macrophage cells were seeded into 24-well plate in density 50,000 cells per well 24 in prior to test; 2 fractions ShS1 in various concentrations (0.1–5 mg/ml) were added to the cells and co-incubated for 2 h. To test cell morphology, the monocytes were seeded onto coverslip glass slide chambers (VWR), treated with ShS1 and stained with DAPI and actin phalloidin effer 760 (eBioscences). The cells were imaged under Olympus IX81 inverted fluorescent microscope. Live/Dead Assay® was carried out according to recommended manufacturer’s protocol (Live Technologies) for fluorescent microscopy. Untreated cells served as negative control.

3. Results and discussion

3.1. Synthesis of ShS1 particles

The optimal process temperature is found to be 50 °C, and time is 45 min. The synthesis is appeared to be sensitive to the ionic strength of process constituents— an increase in Na⁺, K⁺, Cl⁻ caused a failure in the transformation from intermediate amorphous orange Sh₂S₁ into the red or purple crystal ShS1. The intrusion of fillers, either passive (gelatin) or functional (surfactants), has a significant influence on particles micro- and macrostructure. Fig. 1 shows ShS1 synthesized in presence of different side compounds indiffent to main precursors.

A typical ShS1 crystal has a needle shape; individual crystals tend to arrange into spherical rosettes. Many studies have been undertaken to produce variable particle forms and phases: pyramids and rods [4,19], gel [20], needles [2]. Several methods have been applied, including laser fabrication, hydrothermal, single-precursor synthesis, and similar to us, sonochemistry. The main benefit of route suggested here over others, is its simplicity and robustness. The addition of fillers enables control of crystal parameters.

The mechanism of the suggested route was not clearly investigated before; orange amorphous precipitate antimony trisulphide is the intermediate phase of the synthesis. Cao et al. in 2004 [21] previously published work where they discussed the formation of Sh₂S₁ dendrites, which are very similar in crystalline structure to ShS1. They showed that during the transformation of amorphous sulphide to crystalline state, the initial rod formations undergo tip splitting resulting in dendrites. The H⁺ concentration has been pronounced as a crucial factor for the roughness of rods and hence in the stimulating their splitting into dendrites. According to these results, our ShS1 rosettes can be considered as the extreme stage of rod splitting. Since ShS1 and Sh₂S₁ have the same growth direction (001), we suggest the following mechanism of reaction: The Sh₂S₁ colloid is formed first, as the process has lower Gibbs energy than ShS1; however, low pH hinders sulphide transition to a solid state, and the presenting ions intercalate S⁻−Sh chains and thus equilibrium shifts towards more stable product —ShS1. In this model Sh₂S₁ serves as template for ShS1.

Poor control of the kinetics of ShS1 formation is a significant downside of the method. We attempted to solve this problem by spacial separation of the crystal formation centers. Surfactants are known to be able significantly change the crystal growth parameters and, as a result, the structural properties [22]. We used three different types of non-ionic (polyvinylpyrrolidone, Tween 80), cationic (hexadecyltrimethylammonium bromide), and

![Image](https://via.placeholder.com/150)

Fig. 1. SEM images of synthesized products after treatment with different surfactants (from top left to bottom right: Tween 80, L-ascorbic acid, hexadecyltrimethylammonium bromide, L-ascorbic acid, acetic acid, SDS, PVP). ShS1 formation was confirmed by EDX for experiments with Tween 80, L-ascorbic acid and SDS.
anionic (sodium dodecyl sulfate (SDS), ascorbic acid, acetic acid) surfactants. Fig. 2 shows XRD spectra of the made powders. SbSi showed highest affinity to anionic agents; when taking the mechanism suggested above into consideration; negatively charged SDS molecules trap exceeding 100 pm. This facilitates stabilization of initial SbS3 rods by preventing their erosion and subsequent splitting in highly acidic media. Template deterioration becomes a prevalent process through inhibition effects and iodine takes its place in the crystal structure. The same interaction between HCl and SDS was used by Nagamine and Sasaoka in 2002 to make nanoporous template constructions from titanate tetraanoporoxyde [23].

3.2. Investigation of photophysical properties of SbSi particles

The ultrasonic treatment enables one to disperse larger particles into quite uniform smaller size particles. Breaking down small amounts of SbSi (80 mg in 80 ml of milliQ water in presence of citric acid as stabilizer) under high-frequency ultrasound resulted in broadly absorbing nanoparticles. Fluorescence has narrow profile with an emission peak centered on 702 nm wavelength. However, the origin of the fluorescence is unclear and requires detailed study.

3.3. In vitro studies of SbSi particles

Antimony (V) compounds are widely used in leishmaniasis treatment [24,25]. Since mid 1960s the anti-cancer activity of various antimony (III) organometallic complexes has been explored [26,27]. Recently, antimony pentasulfide nanoparticles have been studied for their toxicity to murine peritoneal macrophages and leishmania parasites [28]. Considering that, toxicity of SbSi should be investigated to evaluate particles potential to be used to target quickly expanding cells. RAW264.7 monocyte/macrophage cells were chosen due to very short doubling time, approximately 11 h.

A number of cytotoxicity tests have been carried out for SDS and t-ascorbic acid treated fractions in a range of concentration 100 μg–5 mg (Fig. 3). Untreated RAW264.7 cells were used as a negative control. Elevation in SbSi dosage led to gradual reduction in the cell number and breathing activity. The same dose-dependent effect has been observed in Live/Dead assay. Cytotoxicity is found to be more pronounced for t-ascorbic modified sample.

High short-term dose-dependent cytotoxicity was observed in cell viability tests (Fig. 3). The reason might be due to the residue of sulfide-ions on the crystal surface or due to ions leakage from the particles. The SDS treated crystals showed less toxicity than t-ascorbic acid fraction. Cell morphology remains healthy, except for those exposed to 5 mg/ml – nuclei which appeared to be deformed, with clumps inside; here actin is disrupted and observed as small islands, what refers to high amount of necrotic cells.

The observed difference in cell death is related to the capping of the crystal surface and its morphology. Well-shaped SDS-treated particles contain less residues like unreacted sulfur and antimony. Also as-prepared needles have a smooth surface; this prevents trapping of contaminating ions. Use of t-ascorbic acid in synthesis has resulted in crystal deformation and shift the reaction towards amorphous structures. This leads to an increase in the concentration of impurities which can’t be easily washed off (Cl−, Sb3+, S2−).

![Cytotoxicity tests](image1.jpg)

Fig. 3. Cytotoxicity tests. A/B: Morphology of RAW264.7 cells treated with 5 mg/ml t-ascorbic acid modified SbSi B (lower right), untreated cells A (upper right) were used as control. Nuclei stained with DAPI (blue), actin stained with phalloidin (red). Actin is fully destroyed in treated cells (B), membrane integrity loss is caused by exposed particles. C: Alamar blue viability test. Untreated cells remained as control, dye was used as stain control. Brack bar represents standard deviation value. Fraction 1 is SDS-modified particles. Fraction 2 is t-ascorbic acid treated, respectively. The concentration range tested is 0.1–5 mg/ml. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
4. Conclusion

We have demonstrated that antimony sulphoxide can be synthesized by a quick "one-pot" method in aqueous solution. The mechanism of the reaction was investigated and found to be strongly dependent on pH: high acidity is crucial condition for amorphous Sb2S3 intermediate to be transformed into crystal antimony sulphoxide. However, trisulphide formation is an important stage as it serves as a template for Sb-S particle formation. Both compounds have the same needle-like morphology and growth direction (001). Unless Sb2S3 is more stable thermodynamically, high concentration of H2S leads to its erosion and intercalation by I-.

The shape of resulted crystals can be controlled by inhibiting Sb2S3 deterioration – temporal stabilization of intermediate product improves needle growth and prevents tip splitting to the rods. This can be achieved by doping reaction mixture with anionic surfactants like SDS. These molecules interact with excessive H2S and thus facilitate crystallization.

SDS has been found as prominent particles growth control agent, it's neutral to the precursors and doesn't interfere with the reaction. The SDS modified Sb2S3 has demonstrated all typical traits (needle shape, deep red color, XRD pattern, crystal uniformity), but the dimensions of crystals were reduced.

In vitro cytotoxicity studies have shown that ascorbic acid treated fraction of Sb2S3 demonstrated significant toxicity comparing to the fraction modified by SDS. The reason is the structural difference of the resulted crystals – SDS helps to reduce needles dimensions whilst the surface and morphology remains smooth and constant. Ascorbic acid increases Sb2S3 amorphia and promotes surface defects, hence it creates good conditions for the by-products trapping and contaminating with the residing toxic ions (S2-, Cl-, Sb23+). The tested particles showed fairly high ability to kill fast expanding cells, what can be used in anti-tumor treatment.

Supplementary information

TEM, more SEM images, UV–vis absorption and PL-spectra can be found in Supplementary information section.

Contacts of interests

None declared.

Acknowledgments

This work was conducted under the framework of the Irish Government’s Programme for Research in Third Level Institutions Cycle 5, National Development Plan 2007-2013 with the assistance of the European Regional Development Fund (INSPIRE) and the Ministry of Education and Science of the Russian Federation (Grant no. 14.825.31.0002). Authors are grateful to final year chemistry students A.N. Byrne (BCS) and John Lynn.

Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.matlet.2015.07.027.

References

Effects of Polydopamine Functionalization on Boron Nitride Nanotube Dispersion and Cytocompatibility

This paper was originally submitted for the "Biomaterials: The Third Generation of Medical Devices", published as the July 15, 2015, issue of Bioconjugate Chemistry (Vol. 26, No. 7).

Marc A. Fernandez-Yague,1 Aitor Larrañaga,1,2 Olga Gladkovskaya,1 Alanna Stanley,6 Ghazal Tadayyon,1 Yina Guo,7 Jose-Ramon Sarasua,2 Syed A. M. Tofail,1 Dimitrios I. Zeugolis,7,1 Abhay Pandit,1 and Manus J. Biggs.1,7

1Centre For Research in Medical Devices (CURAM) and 4Regenerative Modular & Developmental Engineering Laboratory (REMODEL), National University of Ireland Galway (NUIG), Galway, Ireland
2Department of Mining-Metallurgy Engineering and Materials Science & POLYMAT, School of Engineering, University of the Basque Country (UPV/EHU), 480130 Bilbao, Spain
3Department of Anatomy, National University of Ireland Galway (NUIG), Galway, Ireland
4Department of Physics and Energy, and Materials and Surface Science Institute (MSSI), University of Limerick, Limerick, Ireland

Supporting Information

ABSTRACT: Boron nitride nanotubes (BNNTs) have unique physical properties, of value in biomedical applications; however, their dispersion and functionalization represent a critical challenge in their successful employment as biomaterials. In the present study, we report a process for the efficient disentanglement of BNNTs via a dual surfactant/polydopamine (PD) process. High-resolution transmission electron microscopy (HR-TEM) indicated that individual BNNTs become coated with a uniform PD nanocoating, which significantly enhanced dispersion of BNNTs in aqueous solutions. Furthermore, the cytocompatibility of PD-coated BNNTs was assessed in vitro with cultured human osteoblasts (HOBs) at concentrations of 1, 10, and 30 μg/mL and over three time-points (24, 48, and 72 h). In this study it was demonstrated that PD-functionalized BNNTs become individually localized within the cytoplasm by endosomal escape and that concentrations of up to 30 μg/mL of PD-BNNTs were cytocompatible in HOBs cells following 72 h of exposure.

INTRODUCTION

The discovery of nanoscale carbon formulations encompassing one-dimensional nanotubes and two-dimensional graphene has resulted in significant advances in the fields of biomaterials and nanoelectronics. Boron nitride nanotubes (BNNTs), like carbon nanotubes (CNTs), have potential applications in both biomedical and nonconventional electronics thanks to their singular structural and physical properties.1–4 Although CNTs have been largely studied as tubular nanostructures for a number of different applications,5–6 BNNTs have only recently been explored as next generation materials.7–9 BNNTs possess a similar physical structure to CNTs; yet possess significantly different physicochemical properties. In particular, despite the remarkable mechanical properties of CNTs, recent studies have demonstrated that BNNTs possess significantly greater shear strength than CNTs;10 moreover, they have been shown to present higher thermal conductivity, superior electrical band gap properties, and are more resistant to oxidation at high temperatures.11

As with CNTs, in order to explore BNNTs as next generation materials it is crucial to develop cheap and facile methodologies for efficient dispersion and functionalization.12 The difficulties associated with BNNT dispersion in water and organic solvents has proved problematic for the generation of BNNT formulations and nanocomposites, and early studies have concentrated on identifying suitable methodologies to address BNNT dispersion.13–16 Initial approaches have focused on similar covalent and noncovalent methodologies used for CNT dispersion.17 Specifically, acid treatment has been applied to add functional surface groups to CNTs; however, this treatment produces irreversible structural changes and decrements in electronic and mechanical properties of the nanotubes.18–21 Alternatively, noncovalent approaches such as the use of amphiphilic molecules for dispersion have been successfully applied for CNT formulations22–26 commonly in conjunction with an ultrasonication process.27–33

In the present work, functionalization of BNNTs with polydopamine chemistry (PD) was explored as a strategy to...
Figure 1. Schematic of the BNNT dispersion and PD-functionalization process. Pristine BNNTs were dispersed in Tris-HCl under probe ultrasonication to yield a 40 μg/mL suspension (A). BNNT suspension was subsequently centrifuged at 5000 rpm and the supernatant retained (B). The BNNTs were incubated with dopamine hydrochloride under agitation for 18 h before centrifugation at 13,000 rpm and washing (C). Pristine BNNTs retain a fibrous sponge-like morphology in aqueous environments following a 3 h sonication process (D). SDHS (0.5 mg/mL) facilitated dispersion of the BNNTs (E) which become functionalized with a stable PD nanocoating following the addition of dopamine hydrochloride (1 mg/mL) for 18 h (F).

Interestingly, BNNTs possess a unique capacity to generate electric fields under mechanical stimulation (piezoelectricity).\textsuperscript{12} and recent studies indicate that the piezoelectric response of BNNTs is higher than that of piezoelectric polymers such as PVDF.\textsuperscript{42,43} In order to take advantage of these properties, BNNTs have been explored as nanovectors for intracellular release of drugs or for the delivery of electrical stimuli;\textsuperscript{42} however, a greater understanding of BNNT cytocompatibility is critical to facilitate further investigation in this area.

The production of BNNTs by different methodologies such as CBD or ball-milling results in the synthesis of BNNTs with large diameters (30–50 nm) and nanotube formulations with a significant persistence of metallic impurities. Conversely, the BNNTs employed in this study were synthesized using a high-temperature pressurized vapor/condenser method (PVC). The BNNTs possessed between 1 and 5 discrete walls, a high aspect ratio (300 m\(^7\)/g), high crystallinity, and relatively small diameters (2–5 nm).
RESULTS

BNNT Dispersion in Tris-HCl Buffer. Figure 1A–1C shows representative images illustrating the process of BNNT PD coating and dispersion in a Tris-HCl solution. BNNTs were originally in the form of a dry sponge (Figure 1D). With the aid of an ionic surfactant, sodium dodecyl benzene sulfonate (SDBS), the dispersion became homogeneous and white-colored after a 3 h ultrasonication process, indicating the efficacy of SDBS for dispersing and debundling BNNT fibrils (Figure 1E). This dispersion was subsequently centrifuged at 5000 rpm at 4 °C for 5 min and the BNNT supernatant was collected for PD coating. After the dopamine self-polymerization reaction, the dispersion was homogeneous and the color turned from white to brown (Figure 1F).

Characterization of PD-Functionalized BNNTs. Low-magnification TEM imaging was performed to assess BNNT
dispersion before and after PD functionalization. Specifically, it was observed that SDBS dispersed BNNT suspensions tended to agglomerate into bundles of 4 to 6 μm in diameter (Figure 2A). In contrast, PD coated-BNNTs were well dispersed and when cast onto a surface, formed a percolated network (Figure 2B).

BNNTs were observed to possess well-defined walls, typically ranging in number from 4 to 6. Moreover, the unaltered hexagonal arrangements of B and N atoms were observable in pristine BNNTs (Figure 3A). The presence of an investing PD coating was confirmed by energy-dispersive X-ray spectroscopy (EDS) analysis and high-resolution TEM imaging. TEM analysis also allowed the direct observation of PD functionalization of BNNTs, where it was possible to identify a homogeneous 1.5-nm-thick coating along the nanotube surface (Figure 3B,C). The presence of a superficial PD layer was further confirmed via dynamic light scattering (DLS) and it was noted that following PD functionalization the mean hydrodynamic radius was increased from 228 ± 3 to 257 ± 4 nm (see Supporting Information S1–S3).

EDS analysis of SDBS dispersed BNNTs indicated B and N peaks of similar intensity, and also the presence of SDBS as indicated by the Na and S signals. In contrast, following PD functionalization, Na and S signals were reduced and an increased C signal was obtained (Figure 3D,E).

The presence of PD coating was also confirmed by X-ray photoelectron spectroscopy (XPS) that indicated significant differences in elemental composition between BNNTs and PD-BNNTs (Figure 4). Specifically, the carbon content (as indicated by the C 1s peak) increased from 46% for pristine BNNTs to 16.9% for SDBS dispersed BNNTs and to 24.9% for PD-functionalized BNNTs.

To quantify the significant effects of PD functionalization on BNNT dispersion, the absorbance profiles of BNNTs, PD, and PD-functionalized BNNTs as a function of the wavelength were quantified (Figure 5). An increase in the absorbance for PD-functionalized BNNTs with respect to SDBS dispersed BNNTs (non-functionalized) was observed. This increase in absorbance was also observed as a function of concentration. Here we assessed absorbance with concentrations ranging from 0 to 0.2 mg/mL. In this manner, it was possible to estimate the final concentration of PD in a PD-functionalized BNNT dispersion following centrifugation. Accordingly, a value around 20 μg/mL was obtained by assessing the absorbance of SDBS dispersed PDs (see Supporting Information S4) and the aid of eq 1. The effects of SDBS coating were further evaluated via UV–Vis analysis (see Supporting Information S5).

Cytotoxicity of BNNTs. Osteoblasts were cultured in complete media containing SDBS dispersed and PD-functionalized BNNTs of increasing concentrations. Figures 6 and 7 show representative fluorescent microscopy images of live/dead labeled cells cultured with non-functionalized and functionalized BNNTs respectively, and corresponding concentrations of 1, 10, and 30 μg/mL acquired after 24, 48, and 72 h.

Quantification of cell viability was also performed as a function of fluorescence absorbance. Live/dead assay indicated that after 24, 48, and 72 h of culture exposure to increasing concentrations (1, 10, and 30 μg/mL) of non-functionalized BNNTs cell viability was significantly reduced relative to that of untreated control situations. However, cell viability was not affected in HOBS cultured with PD-functionalized BNNTs relative to control conditions. Indeed, a viability of approximately 90% was maintained in all experimental groups after 72 h (Figure 8A). Similarly, cells exposed to non-functionalized and PD-functionalized BNNTs were observed to undergo differential metabolic activity and proliferation rates. Critically, non-functionalized BNNTs significantly reduced both cellular metabolic activity and cell proliferation relative to control conditions. This effect was reversed in cells cultured with PD-functionalized BNNTs (Figure 8BC).

In order to assess the cellular internalization of PD-functionalized and non-functionalized BNNTs, biological TEM was performed at day 3 with all BNNT concentrations. It was observed that both non-functionalized (Figure 9) and PD-functionalized (Figure 10) BNNTs became internalized following 72 h of culture and both nano and microscale aggregations of nanotubes and individual nanotubes as single entities were observed to localize to the cell endosomes. Significantly, at concentrations of 30 μg/mL BNNTs become localized to the cell membrane. Evidence of clathrin-dependent endocytosis as an uptake mechanism (Figures 9D and 10D) was observed for both non-functionalized and PD-functionalized BNNTs. Furthermore, evidence was also noted for the presence of endosomal escape (Figure 10C).

**Discussion**

In the present work, PD functionalization was employed to improve the dispersion of pressurized vapor/condenser synthesized BNNTs in aqueous solutions and, at the same time, provide functional surface groups as a proof of concept study for future functionalization strategies. To improve the reaction between individual BNNTs and dopamine, BNNTs were first dispersed in Tris-HCl (10 mM, pH = 8.5) with the aid of SDBS, which has been previously employed to improve the dispersion of BNNTs in aqueous solutions. 0.47

Dispersion was confirmed microscopically with ultra-high-resolution TEM. At low magnification, large BNNT agglomerations were observed in pristine suspensions with few isolated...
Figure 5. Absorbance spectroscopy of non-functionalized BNNT, PD-functionalized BNNT and PD (0.5 mg/mL) (A), and standard curves showing the extinction coefficients (k) for non-functionalized BNNT (B) and Dopamine (C). The correlation of the curve was 99.8% for n = 3 samples.

Figure 6. Representative images of Live/Dead Assay for HOBs treated with methanol as a positive control, and 0, 1, 10, and 30 μg/mL of SDBS dispensed BNNTs after 24, 48, and 72 h. Cytotoxicity was noted in cells treated with uncoated SDBS dispersed BNNTs at all concentrations and all time-points. Green = live, Red = dead. Scale bar = 50 μm.
Figure 7. Representative images of Live/Dead Assay for HOBs treated with methanol as a positive control, and 0, 1, 10, and 30 μg/mL of PD-BNNTs after 24, 48, and 72 h. Relative to cells treated with non-functionalized BNNTs, the observable number of nonviable cells was not significant at all concentrations. Green live, Red dead. Scale bar = 50 μm.

At high magnification, the number of BNNT walls was measured and its integrity was evaluated. Based on our measurements, the number of walls of BNNTs ranged from 4 to 6. Furthermore, the employed dispersion and functionalization process did not damage the integrity of the wall. Although there is extensive literature concerning different methodologies for dispersion and functionalization of CNTs, many of these produce irreversible changes to the nanotube ultrastructure that can negatively affect the intrinsic properties of the nanotubes. The PD layer was clearly discernible with ultra-high-magnification TEM imaging, and a thickness of approximately 1.5 nm was noted. Considering that the backbone of PD is composed predominantly of carbon atoms, the carbon content increase detected by EDS and XPS can be clearly associated with the presence of PD on the surface of BNNTs. Moreover, this PD layer remained stable even after several washing steps, suggesting a strong interaction between the coating and the nanotubes.

Although PD has been widely employed as a functionalization chemistry for various materials in vitro, its mechanism of self-assembly is still under debate and, therefore, the interaction with BNNTs admits several interpretations. So far, the most accepted structure for PD is one composed of dihydroxyindole and indoleidione units linked by C–C bonds between their benzene rings. Similarly, the presence of open chain dopamine units within the polymer has been also demonstrated. Herein, it is hypothesized that BNNTs interact with dopamine molecules in solution via strong π–π stacking forces between aromatic molecules of dopamine and the closed hexagonal structure of BNNTs, as well as via van der Waals interactions between the amino groups of dopamine and the boron atoms of the BNNTs (Figure 11). Furthermore, the hydrophilic −OH and −NH2 groups present in PD may facilitate the dispersion of BNNTs in aqueous solutions.

Unlike BNNTs, carbon derived nanomaterials (including, but not limited to, carbon black nanoparticles, carbon quantum dots, single and multiwall nanotubes) have found a wide range of applications, such as drug and gene delivery, scaffolds for tissue engineering, and as a nanoreinforcement material. However, in order to make direct comparisons between BNNTs and CNTs a number of parameters should be taken into consideration: length, diameter, concentration, wall number (single or multiwalled), and the presence of surface functional groups.

The biological effects of boron nitride nanostructures have not been extensively assessed, and very few studies have focused on the in vitro compatibility of such. However, a significant number of studies have been conducted on the cytocompatibility of CNTs which indicate that the cytotoxicity, cellular, and immune responses greatly vary, with little consensus as of yet with respect to the mechanisms of action. Similarly, the cytotoxicity of pristine BNNTs has been reported at concentrations as low as 2 μg/mL, while other studies have found pristine BNNTs to be highly biocompatible at
concentrations up to 20 µg/mL both in vitro and in vivo. These conflicting results may be explained by the different methodologies employed for the manipulation and purification of the nanotubes that result in different surface properties as well as the presence of impurities. Furthermore, the length and the diameter of nanotubes have been shown to determine the mechanisms of cellular interaction and nanotube geometry has been shown to modulate cellular toxicity and particle fate.

Critically, it has also been proven that the hydrophobic nature of boron nitride based nanotubes facilitates cellular internalization and negatively affects cell viability. Thus, the need for biofunctionalization seems mandatory. In the case of CNTs, noncovalent functionalization with biopolymers such as PD is proven to be an efficient pathway to solve the problems associated with dispersion, and the use of both synthetic and natural chemistries are gaining more attention for the functionalization of nanoparticle formulations including BNNTs.

In the present study we have investigated the biological effects of non-functionalized and PD-functionalized BNNTs of three different concentrations (1, 10, and 30 µg/mL) and incubation times (24, 48, and 72 h), on HOB viability and activity in vitro. As confirmed by a live/dead assay, significant cytotoxicity was observed in non-functionalized BNNTs at concentrations ≥1 µm/mL, a trend not observed with HOBs exposed to PD-functionalized BNNTs. Furthermore, in order to assess the proliferation rate of HOBs exposed to both non-functionalized and PD-functionalized BNNTs, DNA quantification was conducted via Picogreen analysis, which confirmed that although proliferation decreased as a function of time in HOBs exposed to non-functionalized BNNTs at all concentrations, the proliferation rate was maintained in HOBs exposed to PD-functionalized BNNTs. Similar studies conducted by Ciofani et al. have concluded that 10 µg/mL of poly(l-lysine) (PLL) coated BNNT do not negatively influence viability or function in C2C12 cells after 3 days in culture. Furthermore, tolerable BNNT concentrations were increased to 20 µg/mL when functionalized with polyethylenimine. Interestingly, BNNT functionalization with polymer coatings seems to play a critical role in preventing BNNT cytotoxicity.

A recent study by Horvath et al. concluded that both non-functionalized and acid-treated BNNTs are cytotoxic even at low concentrations. In this study, different cell lines (human embryonic cells and lung alveoli cells) were exposed to pristine and acid-treated BNNTs at different concentrations (0.02, 0.2, 2, and 20 µg/mL). Unfortunately, at present it is not possible to decouple the importance of chemical functionalization in reducing BNNT cytotoxicity from other physical properties such as nanotube dimension and shape, which have been proposed as the principal mediator of BNNT toxicity. In particular, a study of Ciofani et al. on gum Arabic functionalized BNNTs has indicated the importance of the length and aspect ratio of BNNT on cytocompatibility. Here it was noted that the short (1.5 µm) BNNTs employed in their study significantly enhanced cytocompatibility relative to the nanotubes employed by Horvath et al., which possessed lengths of ≥5 µm. This duality of nanotube aspect ratio and surface chemistry in mediating cytotoxicity is also confirmed in the...
present study, which utilizes nanotubes with relatively short lengths (1 μm).

Although nanoparticle uptake is reportedly reduced with nanotubes larger than approximately 1 μm,53 elongated particles including boron nitride nanotubes can be internalized via clathrin-dependent endocytosis as demonstrated in a previous study by Ciofani et al. Here neither C2C12 myoblast viability nor myotube formation was significantly perturbed by BNNT endocytosis following 3 days of culture.55 Energetically active endocytic processes may also be responsible for BNNT internalization, particularly of aggregated BNNT clusters or of individualized nanotubes which may be captured, fused, or accumulated within lipid-rich vesicular compartments.56

In the present study it was observed that internalization of BNNTs into HOBs was via endocytosis; however, the uptake of pristine BNNTs at the same dose was significantly lower. Therefore, it is hypothesized that dopamine coating might allow better dispersion at these concentrations and more efficient cell uptake. Interestingly, it was noted in the present study that BNNTs may be able traverse into the cytoplasmic space. This phenomenon has been previously shown with CNTs, which are able to traverse the endosomal membrane by "piercing" and extending into the cell cytoplasm, a process that has been recently described as "endosomal escape".57 However, a lack of observed toxicity suggests that no significant intracellular interaction between tubes and other intracellular compartments occurred after 72 h.

The experimental data of Jin et al.53 demonstrated that the highest uptake of nanoparticles occurs with nanotubes 320 ± 30 nm in length and decreases with longer (660 ± 40 nm) and shorter (130 ± 18 nm) nanotubes. Interestingly, it has also been demonstrated previously that agglomerated or relatively long nanotubes become localized to the cell surface to form a protective coating that prevents internalization. In the present study it was noted that cells exposed to 30 μg/mL of PDAfunctionalized BNNT were associated with aggregates of nanotubes at the extracellular plasma membrane, while BNNT concentrations of 1 μg/mL were well dispersed and significant deposition at the cell surface was not observed.
Figure 10. TEM analysis of cellular internalization of PD-functionalized BNNTs after 72 h. Hobs incubated with 1 and 30 μg/mL of BNNTs (A, B) demonstrated significant BNNT internalization at day 3. It was observed that large nanoscale aggregates (B) and individual nanotubes (C) were localized in both endosomes (B) and/or escaping from endosomes into the cytoplasm (C). Evidence of clathrin-dependent endocytosis was again observed with nanotube aggregates (D). Scale bar = 500 nm.

Figure 11. Schematic representation of the most accepted structure of PD and its interaction with BNNT via strong π-π stacking forces.

CONCLUSIONS

In this study, it has been illustrated that disentanglement by a surfactant assisted ultrasonication process and subsequent functionalization with PD improved the dispersion and stability of BNNTs in aqueous solutions. In addition, in contrast to non-functionalized BNNT dispersions, PD-functionalized BNNTs were not observed to modulate cellular proliferation or metabolic activity. Osteoblasts exhibited no significant cytotoxic effects when cultured with PD-functionalized BNNTs at concentrations from 1 to 30 μg/mL and for up to 72 h of culture. We conclude that PD functionalization can be employed as an effective strategy for the production of well-dispersed and cytocompatible BNNT formulations. These results are in contrast to previous studies, which have reported that cytotoxicity is present in noncoated BNNTs at concentrations greater than 2 μg/mL. Interestingly, we noted that PD-functionalized BNNTs concentrations greater than 10 μg/mL increased the presence of PD-BNNT bundles in solution as a consequence of the ionic strength of media, and became localized to the HOB plasma membrane. Then, it can be hypothesized that highly agglomerated nanotubes may be deposited onto the cell surface acting as a protective layer, which can prevent the endocytosis of isolated nanotubes. PD-functionalized BNNTs at low concentration were internalized by the cell as individual entities. In contrast, low concentrations
of pristine BNNT did not allow dispersion and uptake of individual BNNTs. To evaluate the future biomedical potential of BNNTs and reveal the benefits of these nanoparticles over widely explored CNTs, further research is required, and further investigations into the cellular uptake mechanisms and BNNT interactions with cellular compartments should be at the forefront of this research. We conclude that dopamine coating can be employed as an effective strategy for the production of well-dispersed and cytocompatible BNNT formulations.

**MATERIALS AND METHODS**

Materials. BNNTs were purchased from BNNT, LLC, USA and synthesized using a high temperature/pressure (HTP) method, also called the pressurized vapor/condenser method (PVC). The purity is reportedly approximately 90% by mass containing residual impurities as microdroplets of elemental boron.11 Dopamine hydrochloride was purchased from Sigma-Aldrich (H8502, MW: 189.64 g/mol) and was utilized for BNNT coating. SDS (28995, Sigma-Aldrich) acts as an ionic surfactant for achieving a good dispersion prior to PD coating. Si mesh lacey carbon film grids were purchased from Agar Scientific. Normal Human Osteoblasts (NH/ Ots), and Colonetics Osteoblast Growth Media were purchased from Lonza. Quant-IT PicoGreen dsDNA Assay Kit (P11496), alamarBlue Cell Viability reagent (DAL110) and Live/Dead Cytotoxicity Kit for mammalian cells (3224) were purchased from Life Technologies (Thermo Fisher Scientific).

Methods. Coating of BNNTs with PD. The schematic representation of the BNNT functionalization process with PD is illustrated in Figure 1. BNNTs were originally obtained as sponges composed of dense fibers. Prior to PD coating, BNNTs were dispersed with the aid of the ionic surfactant SDS. 400 μg of BNNTs were mixed with 10 mL of Tris-HCl (pH 8.5) solution containing 500 μg of SDS. This mixture was then sonicated for 3 h (pulse 10 s ON, 2 s OFF) with an output power of 17 W. Subsequently, the homogenized dispersion was centrifuged at 3000 rpm (2516 g) for 5 min at 4 °C to remove BNNT aggregates and impurities and the supernatant was collected. The concentration of BNNTs in the supernatant was 180 μg/mL, determined via spectrophotometric (Varioskan Flash Multimode Reader, UV–Vis) analysis after obtaining the extinction coefficient of BNNTs for determining their concentration (calibration curve with known concentrations of BNNTs is shown in Supporting Information S4 and S5). Dopamine hydrochloride (1 mg/mL) was then added to the dispersion and stirred for 18 h at RT. During this process, the color of the dispersion turned from white to brown due to the oxidation and self-polymerization of dopamine. Finally, BNNTs were collected by centrifugation at 13 000 rpm (17 005 g) and washed twice with distilled water to remove unreacted dopamine. In this manner, pellets of BNNTs were obtained for further physicochemical characterization and cell culture studies (Figure 1). The final PD-BNNT dispersion in DI water was stable for up to 2 months without any precipitation confirmed by DLS measurements. Finally, pellets of SDS and PD-BNNT were directly dispersed by ultrasonication (15 min) in protein free media for further incorporation into cell culture.

Characterization of BNNTs. High-Magnification Transmission Electron Microscopy (TEM). Samples for high-magnification nonbiological TEM were prepared by depositing one drop of a diluted dispersion of BNNTs onto a lacey carbon grid and drying overnight. Samples were examined with a JEDL JEM-2100 F TEM operated at 200 kV using a Field Emission Electron Gun equipped with a Gatan Ultrascan digital camera and an EDAX Genesis XM 4 energy dispersive X-ray (EDS) analyzer. The probe size chosen for EDS analysis was set to 1.5 nm to obtain high-resolution and sufficient signal from the area of interest. TEM was performed in order to both characterize the morphology of BNNT dispersion by measuring the diameter of BNNT aggregations and to evaluate the uniformity of the PD coating.

X-ray Photoelectron Spectroscopy. XPS analysis was performed in a Kratos AXIS Ultra spectrometer using monochromatic Al Kα radiation of energy 1486.6 eV. Low-resolution spectra of B 1 s, C 1 s, N 1 s, O 1 s, and S 2p were taken at fixed pass energy of 20 eV. Surface charge was efficiently neutralized by flooding the sample surface with low energy electrons. Binding energies were determined using C 1 s peak at 284.8 eV as charge reference. For construction and fitting of synthetic peaks of low-resolution spectra, a mixed Gaussian-Lorentzian function with a Shirley type background subtraction was used.

Dynamic Light Scattering (DLS). DLS was utilized to determine the size distribution of BNNTs before and after PD coating with a Malvern Nano-ZS90. Each measurement represents the average value of 10–15 measurements with 20 s integration time for each measurement. All the experiments were carried out at 25 °C in a ZEN0112 disposable cuvette.

UV–visible Spectroscopy. A Varian Cary 5000 spectrophotometer was used to measure the absorbance of the solution. The spectrophotometric values obtained on well-dispersed nanotubes permitted the evaluation of the extinction coefficient. In addition, after PD coating, the measurement of residual PD in solution and onto the nanotube surfaces was realized based on a simultaneous analysis of two-component mixture by UV–Vis. Although it was expected that PD coating can affect the light-absorbing properties of the BNNT, it was possible to quantify the amount of PD within a given range. If the absorption of the light by the two-component additive is considered, then we can determine the amount of PD present by the following equation:

$$A = kC_1 + kC_2$$

where k is the extinction coefficient and C the concentration of each solute. By choosing two different wavelengths, two equations with two unknowns were generated. Note that the two chosen wavelengths can be the same used for obtaining the extinction coefficient (Beer’s Law plot). The chosen wavelengths were 400 and 600 nm (Supporting Information S4).

Cell Culture and Cytocompatibility Assays. Normal human osteoblasts (NH/ Ots, Lonza, USA) were cultured in Dulbecco’s Modified Eagle Media (DMEM; Sigma), supplemented with 10% fetal bovine serum (FBS; Sigma), 100 μg/mL of penicillin, and 100 μg/mL of streptomycin in an atmosphere of 5% CO₂ at 37 °C and media was changed every 2 days. All assays were conducted on cells from passage 5 to 6. A cell suspension was obtained by addition of 5% trypsin with EDTA (Lonza, USA). After flaring and centrifugation, the cells were resuspended in DMEM supplemented medium and the number of viable cells was estimated with Trypan Blue exclusion and a hemocytometer. Next, viable cells were seeded as below for cell viability analysis. Cells were maintained in culture for up to 3 days and cell viability/cytotoxicity tests were conducted at days 1, 2, and 3.
Live/Dead (Cell Viability) Quantification. Viable cells were seeded at a density of 5000 per well in 96-well (n = 5) plates for quantitative analysis and in 4-well glass bottom chamber slide for fluorescent microscopy. Osteoblasts were cultured overnight and BNNTs were added to the well plates/slides after 12 h and incubated for a further 24, 48, or 72 h. Untreated live and dead cells were used as controls for quantitative analysis. To induce HOB death, cells were incubated for 5 min with 70% methanol. Live/Dead Assay (Life Technologies) was used to visualize viable and necrotic cells. After coincubation, samples were washed with PBS and stained with calcein and ethidium bromide from the kit as recommended by the manufacturer. The cells were incubated with the stock solution for 30 min protected from light. The well plates were immediately analyzed with a Varioskan Flash plate reader. Samples were subsequently imaged on an Olympus IX81 inverted fluorescent microscope with 20x and 100x objectives.

Double-Stranded-DNA (dsDNA) Quantification. Quant-iT Picogreen ds-DNA Assay Kit was employed to assess cell proliferation. Cells were seeded in a 96-well plate to a density of 5000 cells per well, 24 h prior to the addition of BNNTs. Different concentrations (1, 10, or 30 mg/mL) of BNNTs were added in complete medium containing 10% FCS and cells were cultured as described previously. After 24, 48, or 72 h in culture the cells were processed via a Picogreen assay according to the manufacturers protocol. Briefly, cell lysis to release the DNA was performed by a freeze–thaw process. Subsequently, 25 µL of the lysis sample was mixed with 25 µL of TE buffer and 50 µL of a working solution of Picogreen dye in 96-well plate. After incubation for 5 min at RT the fluorescence intensity was measured (exc. 435 nm; em. 535 nm) using a Varioskan Flash plate reader.

Metabolic Activity Quantification. AlamarBlue was used to examine cell metabolic activity. Briefly, HOBs were seeded into 24-well plates at a density of 20,000 cells per well. The cells were left to attach overnight in humidified atmosphere, 37 °C, 5% CO₂. After 12 h, BNNTs of concentrations ranging 1, 10, and 30 mg/mL were added to the cells as described previously. Fluorescence intensity analysis was performed on Varioskan Flash plate reader. Untreated cells were used as controls.

Biological TEM Sample Preparation and Image Acquisition. Following 3 days of culture with functionalized and non-functionalized BNNTs at 1, 10, and 30 µg/mL samples were subjected to three washes using prewarmed 0.1 M Na-Cacodylate buffer (37 °C) and fixed for 30 min using 4% paraformaldehyde, 2% Glutaraldehyde in 0.1 M Na-Cacodylate buffer. Following fixation, cells were washed 3 times using 0.1 M Na-Cacodylate buffer and postfixed using 1% osmium tetroxide in 0.1 M Na-Cacodylate buffer for 20 min. Cells were then dehydrated through a graded series of ethanol (30%, 50%, 70%, 90%, and 100%) each 2x 10 min). For the final ethanol step, 1 mL was added to each T25 flask and cells were removed from the flask using a cell scraper and then transferred to 1.5 mL Eppendorf tubes and pelleted at 4000 rpm for 5 min. Cell pellets were incubated in propylene oxide (intermediate solvent) for 30 min and then infiltrated and embedded with TAA/B low viscosity resin according to standard protocol. For orientation purposes semithin sections (1 µm) were cut and stained by toluidine blue. Subsequently, ultrathin sections (90 nm) were cut and left unstained. Ultrathin sections were examined with a Hitachi H-7000 electron microscope fitted with a 1K HamannTutu Digital Camera. Images were captured using AMTVS42 Image Capture Engine software.

Statistical Analysis. All data were analyzed using Minstabs 17. Analysis of variance (ANOVA) with Tukey’s comparisons posthoc tests were performed after confirming the following assumptions: (a) the distribution from which each of the samples was derived was normal; and (b) the variances of the population of the samples were equal to one another. Statistical significance was accepted at p < 0.05.

References


The effect of “Jelly” CdTe QD uptake on RAW264.7 monocytes: immune responses and cell fate study

O. Gladkovskaya, A. Loudon, M. Nosov, Y. K. Gun'ko, G. M. O’Connor and Y. Rochev

Encapsulation of Quantum Dots (QDs) has become an essential factor which regulates particles cytotoxicity, as well as physical and chemical stability. Negatively charged cellular membranes have a great affinity to nanoparticles with surface molecules carrying positive charge, hence creating perfect conditions for fast and aggressive intracellular penetration. The preference for non-charged outer shells is topical in QD design and various applications. In the current paper we develop gelatinisation as a prominent coating approach to create neutrally passivated QDs with improved biocompatibility. We have revealed the trends in particle’s uptake, accumulation, intracellular localisation and retaining time as well as RAW264.7 monocyte cell fate and immune responses. Also the difference in particle endocytosis kinetics and dynamics has been shown to depend on the QD core size. The intracellular QD content along with cell responses at the population level was quantified by flow cytometry.

Introduction

Quantum dots (QDs) are small semiconducting nanoparticles which are composed of a few hundred atoms that leads to quantum confinement effects, high surface-to-volume ratio and consequently to their exceptional optical sensitivity and reactivity. The exposure of a high number of core atoms to the surface of the quantum dot frequently results in leakage of ions from the particle core and associated free radical formation. Thus non-coated nanoparticles are not suitable for any biological application due to their low compatibility with physiological medium conditions and irregularities in optical parameters. Several strategies have been applied to reduce the QD cytotoxicity including incorporation in micelles and covering with polymers (TOPG, PEG), proteins (albumin), amino acids and sulphur-containing compounds (TGA).

Gelatinisation has been explored as an effective approach to significantly increase the particle biocompatibility without reducing its quantum yield and fluorescence intensity. The surface of “Jelly” CdTe QDs has a mixture of functional groups (e.g. amine, carboxyl, mercapto-groups, etc.) due to the nature of gelatin - it consists of fragmented peptides of dehydroxylated collagen, therefore it doesn’t have a regular structure. As a result, gelatinated QDs can be linked to biomolecules (proteins, antibodies, oligonucleotides, drugs, etc.) by multiple paths.

Macrophages serve as antigen-presenting cells (APCs) expressing CD80 and CD86 receptors belonging to the B7 superfamily of genes. These two biomolecules (also known as B7-1 and B7-2) play an important role in T-cell activation by providing co-stimulatory signals. T-cell promotion requires either the presence of T-cell receptors (TCR) or the ligation of CD28 molecules. However binding of CD152 (or so-called CTLA-4, cytotoxic T-lymphocyte antigen-4) opposes T-cell initiation. At first sight it appears that there is no difference between CD80 and CD86 molecules: they are complimentary to the same ligands, expressed by the same cell types and have the same functions. The distinct behaviours of these two proteins affect the T-cell fate. CD86 has a higher dissociation/association ability and shorter activation time, and it preferably binds to CD28 ligands. CD80 has more affinity to CD152 receptors, but it is expressed on macrophages after CD86 triggering. It is intriguing that although a quicker CD86-CD28 interaction results in enhanced T-cell activation, the opposite pair, CD80-CD152, has a higher affinity, hence the amplified silencing effect.
The RAW264.7 murine macrophage-like cell line has been employed in a number of studies due to its quick doubling time, efficacy in internalizing, comparatively easy activation, good host quality for transfection, and expression of an essential set of inflammatory proteins (IL-6, IL-10 and TNF-α) and surface receptors (CD80 and CD86). These adherent cells have monocyte morphology with the potential to be promoted to macrophages under certain conditions, for example when challenged by lipopolysaccharides (LPSs), or in the presence of mannose containing antigens or TLR. This cell line enables a broad use in *in vitro* biomaterial trials for investigating all kinds of cell–material interactions including cell covering adhesion, cell growth, cell detachment, mitochondrial and proliferation activity, and immune and mitosis profiling. Alternatively, the RAW264.7 cell line can be considered as a reasonable first approach for examining the nanoparticle fate when injected into the blood stream, followed by bio-imaging and the final cleavage.

**Materials and methods**

**QD synthesis**

CdTe QDs were synthesised according to a previously published procedure. Briefly, Al2Te3 reacted with sulphuric acid to produce H2Te gas which was bubbled through an aqueous solution of CdCl2, thioglycolic acid (TGA) and 0.3 g of gelatin, with pH at 11. The molar ratio of Cd : Te : TGA was 1 : 0.25 : 1.4. The reaction mixture was then heated under reflux for 2 to 48 hours depending on the desired nanoparticle size. Narrow size distribution fractions were collected via size-selective precipitation using isopropanol.

**UV-vis and PL spectra**

Absorbance was examined on a Shimadzu UV-1601 spectrophotometer; distilled water was taken as the baseline. PL spectra were recorded on a Cary Eclipse spectrometer. All measurements were performed to characterize the optical properties of the nanoparticles obtained.

**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% Fetal Bovine Serum (FBS; Sigma), 100 μg mL⁻¹ of penicillin and 100 μg mL⁻¹ of streptomycin. Macrophages were maintained under a humidified atmosphere with 5% CO₂ at 37 °C.

**Transmission electron microscopy (TEM)**

Monocytes were seeded onto thermosan films (13 mm diameter) in a 24 well-plate. The seeding density was 50,000 cells per well. The cells were cultured for 24 hours; after that, conditioned culture medium was replaced by fresh media containing QDs in an appropriate concentration. Cells were further incubated in the presence of nanoparticles for next 12 or 24 hours as desired. Cells were fixed with 2.5% glutaraldehyde and post-fixed with 1% osmium. The fixed samples were introduced into an ethanol gradient and embedded into low viscosity resin (TAAB, Berks, England). The obtained blocks were trimmed and proceeded to fine section cut. Samples were imaged using a Hitachi H7000 transmission electron microscope.

**ds-DNA quantification**

A Quant-IT PicoGreen dsDNA Assay Kit was used for a precise counting of the cell number in the probe. The cells were seeded in a 24-well plate to a density of 1 × 10⁵ cells per well, 24 hours prior to the experiment. Different types of QDs (either TGA or TGA-gelatin-covered) within a range of concentrations (1–100 nM final concentration) were added to macrophages. After 24 hours of co-incubation, the cells were subjected to the PicoGreen assay according to the protocol.

**Annexin V apoptosis assay**

In this assay cells were seeded to a density of 2.5 × 10⁵ 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. Briefly, the reduced medium was removed and the cells were washed twice with phosphate buffered saline (PBS). Macrophages were harvested by pipetting in fresh media and then were placed in Eppendorf tubes. Cells were washed twice with PBS immediately after harvesting, re-suspended in 500 μL buffer and stained with a viability dye according to the protocol. Afterwards cells were washed with serum-containing buffer. Finally, cells were prepared and stained with the Annexin V Apoptosis Assay Kit (eBioscience) and directly subjected to flow cytometry. All measurements were performed on a 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.

**QD uptake and CD80/86 surface marker expression**

Flow cytometry was used to detect the amount of internalized nanoparticles and to measure the expression of pro-inflammatory receptors caused by exposure to QDs. All measurements were performed on a BD FACS Canto A. In this experiment cells were seeded into 6-well plates to a density of 2.5 × 10⁵ cells per well and left 24 hours to adhere. The next day, macrophages were loaded with red or green gelatin coated QDs within a range of concentrations (1–100 nM final concentration). After 12 hours (for the CD86 study) and 24 hours of treatment (for the CD80 study), the probes were subjected to the assay according to the standard protocol. Armenian hamster IgG and rat IgG2a K were used as isotype controls for CD80 and CD86, respectively. All antibodies and isotype controls were purchased from BioLegend. The standard staining protocol recommended by the manufacturer was employed. APC and FITC channels were used as references for signal
detection. FlowJo software was used for interpretation of results.

Quantification of QDs

The amount of the ingested QD nano-crystals was defined by FlowJo software. At least 10,000 events were recorded per tube. A consistent macrophage population was selected from the light scatter graph, and 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 the population of interest was found from the overlay of two histograms of cells treated with QDs and untreated controls in the reference channel.

Statistical analysis

A two-tailed unpaired t-test has been used to evaluate the statistical significance of the results. The experiments were compared with the control group. The results were recognised as statistically significant if the p-value is less than 0.05; they’re marked with the asterisk symbol (*) in the graphics. All the p-values are given in Table 1, ESI.f

Results

Physico-chemical properties of QDs

The as-obtained nanoparticles have been fully characterized. Table 1 shows the main properties of QDs. Both batches have a 29 nm Stokes shift and similar negative surface charge.

dsDNA quantification by PicoGreen

Only exposure to the highest concentration (100 nM) had affected the cell viability (Fig. 1). The number of cells was reduced to 36–40% compared to untreated cells after 24 hours of co-incubation. It should be noted that the results of the test reflect the number of live cells in the sample on the day of acquisition, regardless of nanoparticle internalisation. The cell doubling time should be added to contributing factors. Exposure to low concentrations (1 and 10 nM) did not affect the cell viability.

QD uptake evaluation by flow cytometry

Flow cytometry was employed as a quick and simple acquisition method for nanoparticle ingestion. It allows quantification of the intracellular amount of QDs at the population level by measuring the change in the fluorescence and light scatter pattern in each individual cell. It’s been shown that either red or green QDs are accumulating in monocytes over time (Fig. 2); the signal intensity in reference channels has doubled from 12 to 24 hours. The fluorescence response is higher for red QDs. As is observed in the PicoGreen assay, the cell number has not been affected when treated with low doses. We speculate that a threshold should be achieved to promote cellular reaction on the introduced species.23

CD80/CD86 surface molecule expression

Monocytes are professional phagocytic cells aimed to ingest and destroy foreign bodies or trigger further signaling and consequent T cell activation. Changes in CD80/86 surface marker expression evokes inflammatory pathway cascades and activation to macrophages. The marker levels were measured with respect to nanoparticle treatment. Fig. 3 depicts the results of the experiment. Both markers were significantly down-regulated when the cells are introduced to 100 nM concentration regardless of the QD size (5.3 nm for red and 2.3 nm for green). Compared to control cultures, the levels of CD86 were elevated (20–40%) in the case of red QDs (p < 0.005) when treated with 1 and 10 nM.

Electron microscopy

The vesicles with trapped QDs are found within the cytoplasm; cells maintain a healthy morphology despite uptake. Fig. 4 shows the obtained TEM images. No obvious hallmarks of apoptosis or necrosis were observed. The nucleus is smooth, and chromatin is not condensed. The only difference between the control and treated cells is the increased number of endosomes. However, the number of cells in the sections is much smaller than that analysed in any other assay.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Optical properties of green and red TGA-gelatin capped CdTe QDs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample name</td>
<td>Absorption, nm</td>
</tr>
<tr>
<td>Green gel</td>
<td>515</td>
</tr>
<tr>
<td>Red gel</td>
<td>660</td>
</tr>
</tbody>
</table>

Fig. 1 ds-DNA content of RAW264.7 cells treated with red and green gelatinated QDs in various concentrations (1–100 nM) for 24 hours. Exposure to low concentrations (1 and 10 nM) did not affect the cell viability. Only 100 nM dose reduced the number of cells to 30%. All experiments were performed in triplicate.
Fig. 2  The uptake rate of green and red gelatinated QDs after 12 (A, B) or 24 (C, D) hours of co-culture with RAW264.7 cells. In both cases the overall dynamics remains the same as observed at 12 hours – high response from 100 nM treated cells, the signal amplification is directly proportional to the time of exposure. All experiments were performed in triplicate.

Fig. 3  The expression profile of pro-inflammatory surface molecules. (A, B) CD86 expression profile for the monocytes treated with red and green gelatinated QDs for 12 hours. The marker undergoes a drastic decrease when cells were exposed to 100 nM dose due to the high level of cell death and apoptotic responses. In most cases, lower concentrations did not affect the surface marker profile. However, 1 and 10 nM of red gelatinated QDs have elevated CD86 expression; but the down-regulation effect at 100 nM is more pronounced. (C, D) CD80 expression profile for the monocytes treated with red and green gelatinated QDs for 24 hours. Due to a similar function, both CD80 and CD86 behave alike which confirms that expression “shutdown” is related to cell damage caused by high dose of QDs, without triggering inflammatory reaction as expected. All experiments were performed in triplicate.
Annexin V apoptosis test

The conditioned cell cultures were examined for the 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. The picture has drastically changed when monocytes received 100 nM QDs. The uptake rate did not change for green gelatin coated QDs. The intracellular content of red particles has increased twice from 12 to 24 hours and reached over 90% (Fig. 5A–D). Then, this green or red positive subset was divided into 4 quadrants to quantify viable, early apoptotic, late apoptotic and necrotic cells according to annexin V/viability dye staining (Fig. 5E–H). The majority of cells treated with green QDs remained alive during both control observations. The number of healthy cells also was constant. When the cells were co-cultured with red nanoparticles, they mainly appeared to be dead by the necrosis pathway.

Fig. 4  TEM images of untreated monocytes (A), cells treated with 100 nM red (B) and green (C) gelatinated QDs. More images are provided in the ES1.

Fig. 5  Uptake rate of green (A, B) and red (C, D) gelatinated QDs incubated with RAW264.7 monocytes for 12 (A, C) or 24 (B, D) hours. Red line in histograms is untreated control, blue line is the experiment (cells exposed to QDs). (A–D) X-Axis is common logarithm of fluorescence intensity in reference green FITC (A, B) or red APC (C, D) channel. Y-Axis is frequency of data distribution. (E–H) Observed apoptosis/necrosis profile after exposure to green (E, F) or red (G, H) gelatinated QDs for 12 (E, G) or 24 (F, H) hours. X-Axis is the common logarithm of the fluorescence intensity in the Annexin V corresponding channel, Y-axis is common logarithm of the fluorescence intensity in a viability dye reference channel. Cellular subsets: Q1: necrotic, Q2: late apoptotic, Q3: early apoptotic and Q4: live cells.
Discussion

In our work we used serum-containing culture media to emulate physiological medium conditions. The formation of a protein corona is one of the key events defying further the cell–particle interaction and can't be neglected.14–17 To reveal the potential effects caused by long exposure to QDs, continuous cell culture is required, which is not feasible in a serum-free environment. Wang18 showed that the ingestion pathway, rate and cytotoxicity are not the same once serum proteins are involved. Rapid intake of small amounts of QDs, by cells other than professional phagocytes, has been shown in a number of studies.19–21 Pulse co-incubation (typically up to a few hours) with bare nanoparticles in solution was performed, with an excellent outcome in terms of a high rate of targeting with no or very little cell death. This system is a good first approach for efficacy evaluation. However “real life” cell targeting and drug delivery have more complex routes than the direct cargo-target contact.

The drastic difference in the uptake kinetics pattern is exemplified in Fig. 5. For green-emitting nanoparticles the uptake rate hasn’t changed from 12 to 24 hours (66 and 67% respectively), or the number of viable cells (93 and 90%). The histogram peak shifts to the right (higher fluorescence), proving QD accumulation over time. As was described by Aberg,22 in continuous exposure to nanoparticles the internalisation is a heterogeneous process and depends on the phase of the cell cycle. According to their study, the intracellular amount of nanoparticles can be ranked as G2/M > S > G1.20,23 Apparently, in long cultures (longer than one cell division cycle) with neutral nanoparticles two processes are competing: accumulation and export. In the case of toxic species a third parameter is contributing, namely cell death and subsequent nanoparticle release to media. According to that, the diagram (Fig. 5B) is the signal from cell accumulated QDs (dividing cells) and the rest of the histogram represents the average response from cells in the S/G1 phase. A small amount of cells goes through the apoptotic (3.2 and 7.2% at 12 and 24 hours) or necrotic pathway (1.7 and 2.2% respectively).

The uptake heterogeneity concept is in striking agreement with the results of the experiment involving red-emitting nanoparticles. If we assume that active QD phagocytosis takes place only in one phase of cell cycle, then, after first 12 hours of QD exposure, we will have a cellular subset with significantly high intracellular content of nanoparticles. Considering that cytotoxicity is a cumulative parameter, this subset is likely to die. In next 12 hours necrotic cells are eliminated from the system. The remaining cells proceed through the proliferation cycle again and accumulate more QDs. It results in a strong fluorescence intensity peak shift to the right and an increase in the number of dead cells.

The QD size contributes to the uptake dynamics. Chithranil and Chan24,25 have found the preferred QD size for efficient ingestion. This has been explained by the dependence of the wrapping time on the diameter of primary vesicles when loaded with nanoparticles. According to the study, the optimal diameter for spherical particles is 50 nm. This result was confirmed by Osaki.26 Nevertheless, when the core size of QDs does not exceed 10 nm, the protein corona increases the hydrodynamic size up to hundreds of nanometers. Similarly, Wang24 and Jiang27 suggested that if only large clusters of nanoparticles are formed locally, ingestion might occur. Apart from that, the mechanism and parameters defying uptake are still under discussion. It has been agreed that uptake is an energy dependent process for particles with a core size of 5 nm and above; smaller dots can be transported passively.28 Red-emitting QDs enter the cells via the clathrin-mediated route;21,27,28 however Zhang and Monteiro-Riviere29 have found the caveolar/lipid raft as the endocytosis-mediated route via the G protein receptor pathway and the low-density lipoprotein (LDL)/scavenger receptor. Also there’s no solidity in the questions whether the surface coating/charge20,21 influences the uptake or makes no difference.30,29 And whether it is more important than the hydrodynamic size or not.30 The observed contradictions may be related to different cell types used in the experiments.

Conclusion

In the current study we investigated the behaviour of gelatin coated QDs under serum-containing conditions and their interaction with the cells in continuous cultures. Following earlier research, suggesting distinct patterns from that in protein-free media,16 elevation of the toxic dose (100 nM over 10 nM in previous studies) and different cellular responses to the exposed dots of various sizes (2.7 and 3.7 nm) for a time greater than the cell cycle were confirmed. Our results suggest that heterogeneity in the pace of uptake depends on the cell cycle phase. Unlike pulse treatment, where QDs were co-incubated for a short time and particles were captured regardless of the cell cycle phase, in our study it’s one of the contributing parameters in endocytosis kinetics.

Only 100 nM concentration is considered to affect the cell function. Surprisingly, surface marker expression levels have dropped down to less than 50% from the control. Either green or red QDs drastically decrease the cell number at 100 nM concentration. In the case of red QDs massive cell death via necrosis was observed; this occurred with twice the uptake rate at the 24 hours acquisition point (from 50 to almost 100%). Overall, both QD types tend towards an intracellular occupancy and have a longer retention time when compared with less passivated particles. The ingested nanoparticles form conglomerates and are trapped into the endosomes, clearly observable in the cytoplasm (Fig. 4).

Funding sources

This work was conducted under the framework of INSPIRE, the Irish Government’s Programme for Research in Third Level Institutions Cycle 5, National Development Plan 2007–2013
with the assistance of the European Regional Development Fund, the Science Foundation Ireland (SFI 12/IA/1300 project) and the Ministry of Education and Science of the Russian Federation (Grant No. 14.B25.31.0002).

Conflicts of interests

None declared.

Acknowledgements

The authors are grateful to Shirley Hanley (PhD, NCBES) for help with flow cytometry experiments and Pierce Lalor (Anatomy Department, NUIG) for support with TEM processing and imaging.

References


Chapter 5: Conclusion
Summary

We believe, that all of our published peer-reviewed papers have made an impact to biomaterials research. The conducted studies covered *in vitro* testing of biomaterials and biomedical devices, controllable synthesis and characterization of novel nanomaterials, and multi-parameter intracellular acquisition of fluorescent QDs. Also it has been attempted to build up and develop theoretical approach to predict QDs cytotoxicity in mathematical model based on experimental data. It’s worth to note, studies that incorporate quantitative modelling of nanoparticle cytotoxicity are rare and hence, our concept makes a valuable contribution to such important topic.

In our work we have performed comprehensive study of QDs uptake dynamics and kinetics by monocyte/macrophage cells in physiological media conditions depending on particles size, composition, concentration and exposure time using flow cytometry as quantification method. Number of cellular and immune responses was measured at the same time by multi-color approach. Flow cytometry has been shown as quick unbiased evaluation method of ingested particles on population level. We explained sudden drop in surface markers expression related to QDs uptake and cell function profile. Some previous reports did not outline this phenomenon nor make any hypothesis. Over all, design of majority experiments did not include serum proteins in culture conditions. However, nanoparticle-serum interaction makes a dramatic difference, up to change in ingestion mechanism. Also we have demonstrated that even small variations in QD size (2.3 nm vs. 5.3 nm) lead to ultimately different outcome of cell-nanoparticle interaction.

To explore potential candidates for NIR emitting nanoparticles, which are desirable for non-invasive deep tissue *in vivo* imaging, we have tested antimony sulpho-iodide. It’s ternary semiconductor with outstanding set of physical and chemical properties. Currently it has found application in nanophotonics. The material suffers from lack of easy synthesis method. To address this issue, we developed water-based ultrasonic synthesis of micro-scale SbSI particles to bring it down to nano-scale level. We investigated on reaction mechanism and revealed the role of intermediate product – antimony trisulfide. It has been shown, that Sb$_2$S$_3$ serves as template for SbSI crystals. Also was made successful attempt to control crystal growth; particles with various dimensions were obtained. As-prepared micro- and sub-micro crystals were broken down to nanoparticles with broad absorption. The potential cytotoxicity of different
fractions was tested on RAW264.7 monocyte cell line. Likewise in case of QDs, surface modification of cardiovascular stent played the crucial role in cellular responses. Intriguingly, monocytes and fibroblasts have showed the same trend in preferences regardless alloy composition. Laser surfaces ripples have not attracted monocytes, which is beneficial for implant (hence it prevents inflammatory response and stent rejection). At the same time, ripples were preferred by fibroblasts, what increases possible risk of scarring. The main challenge of this study is necessity to perform cell culture, bioassays, and imaging on 3D non-transparent to light object. This is technically difficult task, as we had to deal with very narrow, curved metal surfaces. It’s resulted in very small number of cells able to adhere to the stents. Besides, majority of bioassays are based on fluorescence, which requires full transparency of light pathway. That was limiting factor in the choice of available methods we could use to characterize cell attachment, growth, and viability. To our best acknowledge, there’re not many papers that tested actual biomedical devices in vitro. Usually the studies were carried out on flat samples, which have different properties, as we have demonstrated in our work. The best example is our attempt to culture endothelial cells on stents and metal coupon. While cells showed excellent growth on flat samples, there was observed poor adherence to stents of the same alloy. In conclusion, this study has reported valuable results of pros and cons of surface laser treatment that can be applied to biomedical devices to extend implants lifetime.

**Conclusion**

1. Developed systematic approach for multi-parameter CdTe QD acquisition by flow cytometry. We have established strategy for intracellular QD quantification and various effects on cells function in cell populations. Investigated parameters include inflammatory surface markers profile (CD80 and CD86), distribution of apoptotic and necrotic cells in respond to QD exposure and uptake. To our best knowledge, this is the first work, which explored flow cytometry for simultaneous QD quantification and broad spectrum of cellular responses.

2. Found the influence of QD size and protective shell composition on cell fate. The outcome of nanoparticle-cell interaction strongly depends on particle uptake kinetic and dynamics. The small (2.1 nm) particles capped only with TGA have
fast internalizing and short intracellular retaining time. High uptake of green TGA-covered QDs promotes cell death via apoptosis. Green TGA-gelatin capped particles remain detectable longer than cell cycle length. Red TGA-gelatin covered QDs are proven to accumulate inside the cells over time with 98% uptake rate after 24 hours of co-culture. This resulted in massive cell necrosis.

3. Explained sudden drop in CD80/86 inflammatory markers expression in cells exposed to 100 nM QDs. This happens due to high QD uptake rate. Intracellular accumulation of nanoparticles causes in turn loss of cell function. The impaired cells are supposed to be eliminated from the system by apoptosis or necrosis. Thus, those cells are not able to maintain normal level of the surface markers.

4. Suggested mathematical approach to predict cytotoxic effects of QDs. The model is based on experimental data obtained in flow cytometry study. To our best acknowledge, this is one of the first theoretical approaches aimed to predict toxic effects of engineered nanoparticles in dependence on their uptake rate. The main advantage of this model is that it can be applied to various cell lines and nanoparticle types. Evaluation of the nanoparticle uptake kinetics and dynamics helps to predict in vitro behavior of a new engineered material. It enables experiment design and optimization without numerous trials. Thus it is easier to find the effective concentration of nanoparticles for various tasks (live cells targeting and tracking, anti-cancer therapy).

5. Found trends in cellular responses to modified metal surface: 3D vs. 2D, and bare vs. laser ripples texture. The relevance of this work is that it deals with real medical devices. Were used stainless steel and platinum-chromium alloys. RAW264.7 monocytes showed high affinity to smooth metal surfaces, whereas MS-5 fibroblasts were better adhered to ripples. Both tested cell types have showed excellent viability on flat metal coupon regardless surface texture. In case of 3D stents, made of the same alloys and possess same surface topography, clearly distinct cell behavior is observed. This shows the influence of the available growth area and stent curvature on cell adherence preference.

6. The aqueous sonosynthesis of SbSI was modified for production of sub-micro sized particles with 100-200 nm thickness. The crystal growth control is improved by use of anionic surfactants. Initially this synthetic route was
developed for bulk production of large bundles of SbSI needles. Our improvements help to expand material use to nanophotonics applications.

7. For the first time has been tested toxicological effect of SbSI on RAW264.7 murine monocytes. Were tested 0.1 – 5 ug/ml range of concentrations; the reduction in cell number and morphological changes were observed in the cells exposed to 1 and 5 ug/ml of SbSI, lower concentrations 0.1 and 0.5 ug/ml have cause mild reduction in viability.

Directions for future work

Flow cytometry is a perfect tool to describe cell population behaviour basing on signal harvested from individual cells. In a combination with mass spectroscopy it gives a unique opportunity to perform unbiased quantitative assay and evaluate exact number of QDs inside the cells in population of interest. As next logical step, we suggest to evaluate intracellular content of QDs by mass cytometry. Mass cytometry method will allow validating the accuracy of detection of fluorescence produced by QDs in flow cytometry analysis. At the same time it will help to reveal the influence of nanoparticles aggregation on signal intensity. Thus it makes possible to build up the correlation between flow cytometry and mass spectroscopy results and expand our knowledge on QDs optical, structural and chemical alterations after ingestion by the cells. Taken together, it will create an effective platform for further implementation of nanoparticles in pharmaceutical and clinical imaging.

Mass cytometry experiments will be also beneficial for further development of mathematical model of QD cytotoxicity. Knowledge of precise intracellular QD content will help to make the model more detailed and informative. In turn, it will enable prediction of toxicity of other engineered materials by expanding existing model to other cell and nanoparticles types.

Apart from classical mathematical approach, quantum chemistry modeling will reveal some nature of forces lying in foundation of observed phenomena. The simulations of QD aggregation effect on fluorescence signal will enlighten what we really see in flow cytometry inside the cells. Apparently, the photoluminescence we measure in colloid QD solution is not the same after interaction with cells in physiological media.
conditions. These calculations will add ultimate accuracy to mathematical model and understanding of nanoparticles toxicity and quantification principles.

Unless flow cytometry has proved its efficiency for QD quantification, there’re many aspects remaining unresolved. For instance, the accuracy of suggested subset selection scheme still requires further validation. On that purpose, cell sorting technique should be included in experimental setup. After the samples been treated with QDs, harvested and stained for flow cytometry, only highly fluorescent cells should be selected and processed for the next step. By comparing cell sorting results to previously done routine cytometry, the efficiency of QD quantification method can be evaluated and improved. Also cell sorting will fulfill the missing details in cell fate after exposure to QDs.

As many researches showed, cell lines respond in different way rather than primary cells. To cover this gap and get one step closer to clinical application, same experiments, as described in current work, should be performed on animal and human PBMCs. It will be a good link between in vitro and in vivo experiments, as well as expand our knowledge on animal model applicability limit.

For better outstanding the effect of nanoparticles on immune system, we would suggest following in vivo study. Mice may or may not be sensitize with ovalbumin, followed by inflammatory response acquisition. After that, the animals will be injected with QDs. The resulted immune responses will be measured in organs and blood. Perhaps, the true effect of QDs lies outside our vision.

Alternatively, ability of QDs to influence and exacerbate inflammatory process can be studied in ex vivo cultures implied in vivo. Briefly, PBMCs can be extracted and cultured in presence of QDs or without them. Then, exposed and naïve cells can be injected back to donor and immune reaction acquired.

In summary, future research in QDs should be more focused on analyzing of gained to-date experimental information and development of mathematical and quantum chemistry models. The understanding of fundamental mechanisms driving QD optical and structure alterations will push further the frontiers in biomedical tools design.