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Cellular mechanisms of transcriptional regulation of human cell lines exposed to cadmium-based quantum dots†

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In recent years, the interest in quantum dots (QDs) has spread across different branches of biology and medicine thanks to their photophysical properties, which make them excellent candidates for use in bioimaging, drug delivery, theranostic applications and, more recently, gene therapy. With the continuous expansion of applications, QD-mediated cellular responses have become of concern. The immune system and the liver have been confirmed to be important targets, and both are sensitive to cadmium sulfide quantum dots (CdS QDs). Here, the effect on mRNA has been studied by whole-transcriptome analysis in human HepG2 cells (as a model of liver cells) and THP-1 macrophage-like cells, and the mechanisms of mRNA regulation by miRNAs during exposure to Cd as CdS QDs or Cd(II) (as $CdSO_4$ 8/3-hydrate) are discussed. CdS QD exposure induced modulation of the transcriptome of hepatocytes activating RAS signaling and increasing intracellular calcium, which results in the activation of the apoptotic pathway. CdS QDs also affect macrophages inducing production of TNFα and other cytokines and hindering the autophagic process. The results obtained *in vitro* on mRNA regulation are partially consistent with those hypothesized after *in silico* analysis of a wide range of miRNAs regulated in the same conditions.

Environmental significance

CdS QDs are nanomaterials with such increasing world-wide diffusion as to raise concerns of environmental and health safety. Environmental concerns increase with the presence of the nanomaterial at sites of production, use or disposal. Health implications derive from environmental diffusion and the intrinsic toxicity of the nanomaterial (to be established yet). Analysis of effects on human cells offer the better opportunity for assessing some of the mechanisms involved. A mechanistic interpretation of the functions involved was obtained after a mRNA–miRNA transcriptomic analysis. The processes involved resulted to have some mechanisms in common with other environmental pollutants and with viral aggression. The instance may offer the opportunity to define from the adverse outcome pathways involved new markers of exposure of both cellular and environmental significance.

Introduction

Quantum dots (QDs),[1,2] with their unique chemical–physical properties, are valuable in a range of commercial and consumer technologies. Their production and usage are increasing, which increases the probability of these nanoparticles entering the environment at various phases of their life cycle.[3] It is therefore necessary to consider their effects on health and environment, considering them as a potentially hazardous material.[4,5] For most applications, release into the environment is mainly expected to occur during QD synthesis.[3] Cadmium (Cd)-based quantum dots (QDs) are commonly used in solar energy cells[6] and, therefore, their dispersion may eventually lead to food and environmental pollution[7,8] (ESI† S1.2).

Because of their ability to enter the human body *via* inhalation and skin penetration, and subsequently to interact with cellular and intracellular structures, concerns over their potential toxicity to both workers and end-users have been raised.[9]

One of the goals for a sustainable nanoindustry is to inform and design for safe and sustainable materials. 'Safe by design'

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is aimed to minimize the potential impacts to human health and the environment.[10,11] After over a decade of nano-safety research, it is indisputable that the vast majority of nano-sized particles induce a plethora of adverse cellular responses – the severity of which is linked to the material's physicochemical properties.[12] Resolving the safety issues associated with this kind of nanomaterial will not only be beneficial to industry, but will also allow and promote many additional applications to the biomedical field.[13] While the potential toxicity induced by the eventual release of metal ions has not yet been definitively established, recent papers on the physical environment of Cd atoms in QDs have excluded this phenomenon as a major determinant of toxicity.[14,15] Previous studies showed that QDs effects on biological material was mainly dependent on the characteristics of the particles as a whole, not on their metal core; these effects are known as nano-effects in the sense that nanotoxicity is discernible from the toxicity of the metal ion.[16,17]

Many studies have shown that, even doses with no overt cytotoxicity, QDs still may cause adverse effects in various cell types.[18,19] The cell response to Cd QDs seems to be mediated by i) the genetic background of the cell and ii) epigenetic effects. Genetic background exerts influence through a series of genes whose absence determines hypersensitivity to the Cd QDs.[20] In plants (*A. thaliana*) three mutations were identified which determined tolerance to Cd-based QDs but not to the Cd ion.[21] More recently, attention has been paid to epigenetic changes induced by QDs and epigenetic mechanisms underlying observed cytotoxic effects in human cells.[22] Epigenetics, including expression of miRNAs, constitute an important link between genotype and phenotype, and play a critical role in the regulation of many cellular processes, such as gene expression. Again, plants provided important clues on the role of miRNA and of mitochondria and chloroplast DNA replication.[23,24]

In our previous studies, we have extensively analysed the cytotoxicity of cadmium sulfide quantum dots (CdS QDs) in human cellular models: hepatocellular carcinoma HepG2 cells and the human macrophage-like THP-1 cells.[16,25] In addition to liver cells, we chose macrophages as targets because the QDs in the human environment can also affect the intestinal immune system after ingestion.[26] Indeed, macrophages,[27] along with lymphocytes,[28] are the most common immune cell models to evaluate QDs toxicity *in vitro*. However, macrophages and monocytes can actively take up QDs, but lymphocytes do not.[29] Phagocytic activity and preeminence in innate immunity have rendered macrophages a very attractive model for studying interaction with QDs.

Using HepG2 and THP-1 cell lines, as an experimental system, and the profiling of a wide series of miRNAs, we have shown that cellular response to CdS QDs is related to the basal activity of cellular signalling pathways and that the two cell lines respond in different manners. Macrophages appear to be less susceptible to toxicity than hepatocytes, even though they accumulate QDs more readily. The transcriptomic approach used until now has allowed us to identify differential changes in miRNome in response to QDs.

As we have previously observed, cellular response to CdS QDs usually consists of changes in the expression of miRNA linked to apoptosis and autophagy. miRNAs can repress (but in some cases also activate) their target genes and degrade the mRNA targets by reducing the mRNA translation efficiency, re-organizing nuclear structure, and remodeling chromatin. This epigenetic cross-talk between miRNAs and mRNAs was modelled with an *in silico* approach.[30] However, the link between miRNA regulation and their potential targets (mRNAs) may concern both transcription and translation of a specific gene and in the different or opposite way.[12,31] The *in silico* previsions were challenged here with an *in vitro* mRNA transcriptome analysis. mRNAs modulated after exposure to Cd QDs were analysed by whole-transcriptome sequencing (RNAseq). *In vitro*/*in silico* interaction/regulation between mRNAs and miRNAs differentially expressed in response to Cd as CdS QDs or Cd(II) (as $CdSO_4$ 8/3-hydrate) was studied and modelled to obtain a new and more supportive view of the adverse effects of the treatments.

Results and discussion

Treatments with CdS QDs and Cd(II)

In a previous study, the regulation of miRNome in two human cell lines exposed to different doses to various levels of Cd, either CdS QDs or Cd(II) was described.[25,30] This study showed that the macrophage line THP-1 is less susceptible than the hepatocytic line HepG2, even though the former accumulates QDs more readily than the latter. HepG2 cells seem to head towards apoptosis when exposed to CdS QDs, whereas THP-1 cells readily move to autophagy. Moreover, it was shown that the response to Cd-based QDs was quantitatively and qualitatively distinguishable from the response to Cd(II).[25,30]

In the same papers, there were *in silico* insights about regulation of miRNAs and of the putative target miRNAs. In this paper, these *in silico* conclusions were submitted to an *in vitro* experimental validation through massive RNA sequencing. Thus, both cell lines were exposed to sub-toxic doses of QDs, mRNAs were extracted and sequenced to define the relative mRNA regulation. In particular, HepG2 were exposed to 2.3 μg ml^{-1} Cd as 3 μg ml^{-1} of CdS QDs and 5.2 μg ml^{-1} of Cd(II); while THP-1 cells were exposed to 5 μg ml^{-1} Cd as 6.4 μg ml^{-1} of CdS QDs and 11.4 μg ml^{-1} of Cd(II) and to 39 μg ml^{-1} Cd as 50 μg ml^{-1} of CdS QDs; in both cell types the concentration of CdS QDs was sub-toxic (Table S3†).[16,30]

THP-1 cells treated with 6.4 μg ml^{-1} of CdS QDs did not show significant modulation in mRNA expression. This treatment does not seem to induce a specific cellular response in accordance with what was shown in previous analyses of the miRNome.[30] Therefore in the following discussion we will refer only to the treatment of 50 μg ml^{-1} of CdS QDs for THP-1 cells.

mRNA regulation in HepG2: miRNA–mRNA interaction, interplay, and adverse effects

In HepG2 cells exposed to CdS QDs, RNASeq experiments gave a total of 1947 differentially expressed genes (DEGs), including

1037 up-regulated and 910 down-regulated genes (Fig. S1a and Table S4†). A total of 4586 DEGs were recognised in HepG2 cells exposed to Cd(II), 1359 up-regulated and 3227 down-regulated genes (Fig. S1a and Table S5†). The analysis demonstrates that exposure to CdS QDs affected the HepG2 transcriptome differently from Cd(II). The principal component analysis (PCA) of mRNA profiles clearly distinguished treatment with CdS QDs from that with Cd(II) (Fig. S2†).

In particular, the Gene Ontology (GO) biological process enrichment analysis conducted on the mRNAs of HepG2 cells exposed to CdS QDs showed a set of biological processes mainly associated with calcium ion transport, xenobiotic glucuronylation, and membrane potential. Exposure to Cd(II), instead, showed the up/down regulation of mRNAs involved, not only with glucuronylation events, but especially with management of DNA damage and cell cycle regulation (Fig. S3 and Tables S8 and S9†).

Therefore, analysis of RNAseq data in HepG2 cells exposed to CdS QDs did not show the regulatory changes in all mRNA involved in apoptosis as suggested by the *in silico* study based on miRNA profiling.[30] The most likely consideration was that the sub-toxic dose of QDs utilized did not affect the mRNA regulation as promptly as with miRNAs (Table S1†). Nevertheless, in analysing one of the principal pathways involved in the cellular response to QDs, the activation of the RAS signalling pathway emerged. This pathway is sensitive to intracellular calcium (Ca^{2+}) variation, leading to the activation of one of the effectors of the pathway, the small GTP-binding protein Ras, which regulates cell proliferation and differentiation.[32] Considerations on the mRNAs–miRNAs interactions involved in this pathway indicated the activation of the calcium signalling pathway could increase intracellular Ca^{2+}.[32] The genes encoding for Ca^{2+} channels (as CACNA1C and CACNA1B, in Fig. 1 CAV1 and CAV2), involved in

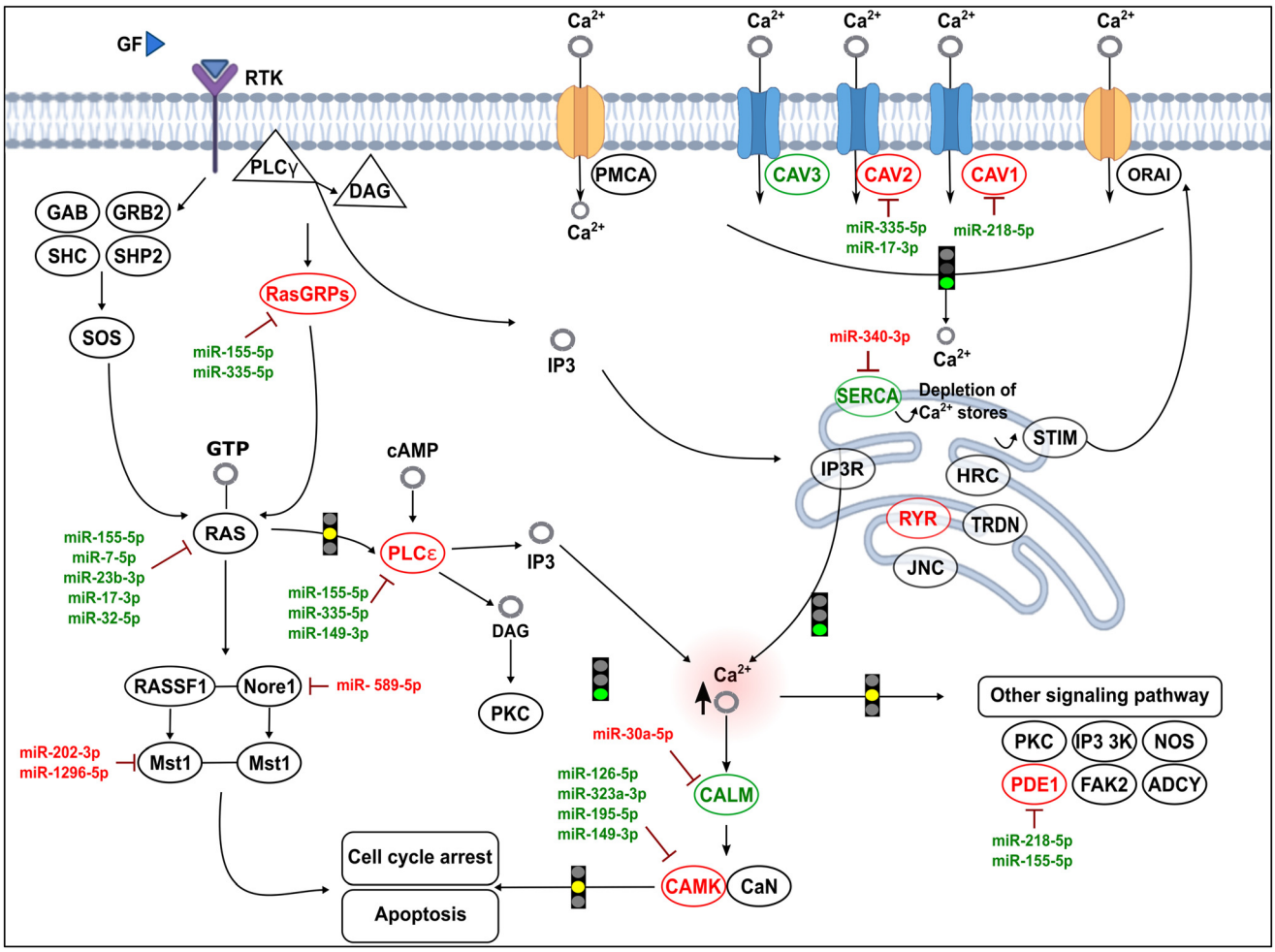

Fig. 1 Main mRNA–miRNA regulation in HepG2 cells treated with 2.3 μg ml^{-1} Cd as 3 μg ml^{-1} CdS QDs. The figure depicts events in RAS and calcium signalling pathways. Genes CAV1 and CAV2, encoding for Ca^{2+} channels, are up-regulated. RASGRP family genes are up-regulated and they activate Ras. This in turn regulates the phospholipase C activity (PLCε gene is up-regulated); IP3 is derived from the action of this phospholipase. This process is related to high Ca^{2+} levels; the increase in intracellular Ca^{2+} triggers the activation of CaMK which results in the activation of the apoptotic machinery. mRNAs or miRNAs that changed their abundance in response to the treatment are showed in red (increase) or green (decrease). As a result, the specific step is unlocked (green light) or could be unlocked (yellow light). DIANA-Tarbase database, DIANA-mirPath and KEGG were used to create this chart. Some icons were created with **https://BioRender.com**.

regulation of intracellular calcium flux, are up-regulated, whereas miRNAs that regulate them are down-regulated (Fig. 1). Therefore, the increase of intracellular Ca^{2+} may eventually lead to apoptosis in the HepG2 cell line.[33,34] As a matter of fact, many of the functions of the liver are regulated by increasing intracellular Ca^{2+}. These include: i) glucose and energy metabolism through the modulation of regulatory enzyme activity and changes in mitochondrial Ca^{2+}; and ii) control of the cell cycle, which includes modulation of transcription[35] and synthesis/activation of anti- and pro-apoptotic proteins[36,37] to regulate cell proliferation and death, which in turn, is regulated by nuclear Ca^{2+}.[38]

In particular, RAS guanyl nucleotide-releasing protein (RASGRP) family genes are up-regulated; they function as a diacylglycerol (DAG)-regulated nucleotide exchange factors that activate Ras through the exchange of bound GDP for GTP (Fig. 1).

This in turn regulates the phospholipase C activity. In fact, the PLCε gene is over expressed and the miRNAs, which regulate it, then down-regulate the PLCε gene. The phospholipase catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate to generate two second messengers: inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). This process is related to high Ca^{2+} levels (Fig. 1). DAG remains at the plasma membrane level to activate some isoforms protein kinase C (PKC), while IP3 diffuses into the cytosol to bind to the inositol 1,4,5-triphosphate receptor (IP3R), causing receptor opening and subsequent Ca^{2+} release from its intracellular stores.[38,39]

RYR1, a receptor on the endoplasmic reticulum which provides an increased pool of Ca^{2+} for a positive feedback on IP3R,[40] is overexpressed in HepG2 cells. These channels are activated by Ca^{2+} through a feedback mechanism known as Ca^{2+}-induced Ca^{2+} release (CICR). The ability of Ca^{2+} to stimulate the RYRs depends on cyclic ADP ribose (cADPR).[38,39]

The increase in intracellular Ca^{2+} triggers the formation of the Ca^{2+}/calmodulin complex, as monitored in intact cells,[41] followed by functional modulation of the target proteins.[42]

Calmodulin (CALM) plays a fundamental role in amplifying Ca^{2+} signalling. To amplify the signal generated by Ca^{2+}, CALM activates a protein kinase dependent on Ca^{2+}/CaM (CaMK), initiating the activation of the substrates.[43,44] CaMKs are master regulators that modulate a space-temporal complex dynamic of multiple activity-dependent protein phosphorylation events during gene expression, with cytoskeletal remodeling, and critical control over a wide range of biological functions.[43]

One function of Ca^{2+} is to control the expression of apoptotic signalling components such as the Fas system. In addition, Ca^{2+} can also induce apoptosis in response to various conditions; this often depends on an interplay between the mitochondria and the ER,[39,45] creating a continuous ebb and flow of Ca^{2+} between these two organelles. There are indications that pro-apoptotic stimuli influence how mitochondria respond to this periodic flux of Ca^{2+}. The Ca^{2+} signals produced by IP3 are normally handled but, in the presence of a stress signal, they induce to apoptosis *via* opening of the mitochondria permeability transition pores (mPTP). These structures are usually formed when mitochondria become overloaded with Ca^{2+} and lead to the release of cytochrome c.[39,46]

Thus, in HepG2 cells, CdS QDs would deplete the endoplasmic reticulum (ER) Ca^{2+} stores, inducing, directly, the opening of plasma membrane Ca^{2+} channels and an ER stress response which result in the activation of the apoptotic machinery.

The Cd(II) response was completely different: after exposure to ionic Cd, cells undergo a stressful condition involving the activation of p53 signalling pathway. Despite the high levels of p53, the low Ca^{2+} concentrations do not cause a stress which blocks the cell cycle. Indeed, p21 is down-regulated and the miRNAs that regulate it are widely up-regulated (Fig. 2).

In general, exposure to Cd(II) caused a general down-regulation of mRNAs and miRNAs (Fig. S1a†). This may determine a shutdown of many cell functions. In fact, the few up-regulated genes were involved in the general and oxidative stress response (GSTM1, GSTTA1, cytochrome p450 family genes).

At variance with the response to CdS QDs, which had a basic effect on Ca^{2+} signalling pathway, the cellular response to Cd(II) does not involve the calcium signalling pathway.

mRNA regulation in THP-1 cells: miRNA–mRNA interaction, interplay, and adverse effects

A total of 2566 DEGs were identified in THP-1 cells exposed to CdS QDs, which included 838 up-regulated and 1728 down-regulated genes (Fig. S1b and Table S6†). Exposure to Cd(II) showed 2490 DEGs, of which 935 up-regulated and 1555 down-regulated genes (Fig. S1b†). Principal component analysis (PCA) of mRNA profiles in the two conditions clearly distinguished treatment with QDs from that with Cd(II) (Fig. S2 and Table S7†).

Furthermore, 520 genes were identified common in two cell types when exposed to CdS QDs, of which 263 had a contrasting behavior in HepG2 and THP-1 cells (Fig. S1c†). In addition, 720 genes were identified common to both cadmium treatments and 433 genes responded in opposite manner in the two treatments (Fig. S1d†).

In general, there was a major down-regulation of DEGs in THP-1 cells exposed to CdS QDs as compared to HepG2 cells. This massive change could not be solely a problem of higher dose because it was sub-toxic, similar to the dose used for HepG2 cells.

In particular, the GO biological process enrichment analysis of THP-1 cells exposed to QDs showed a set of biological process mainly associated with the JAK/STAT signalling pathway, which is involved in mediating cellular responses to cytokines, inflammatory responses and calcium signalling (Fig. S4a and Table S10†). Exposure to Cd(II)

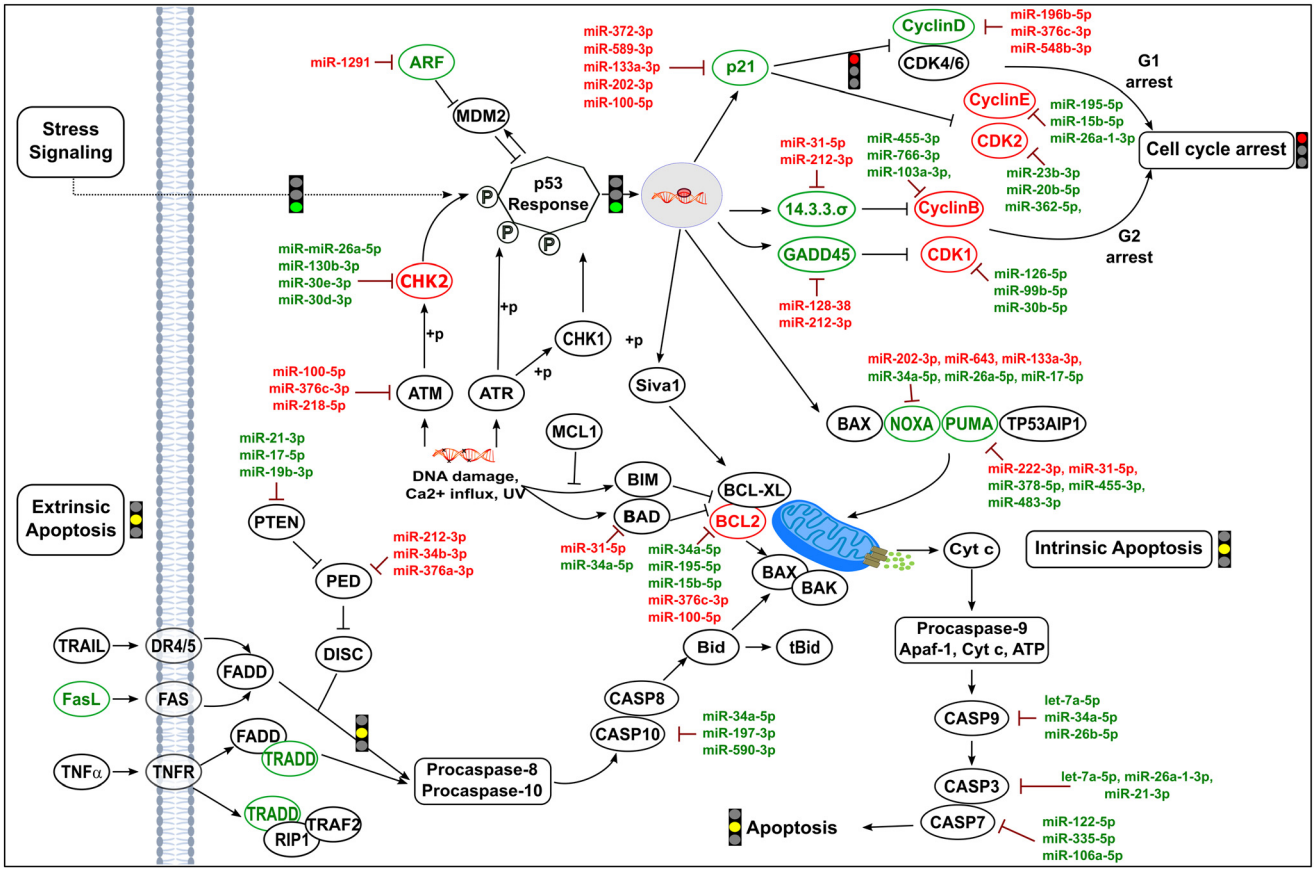

Fig. 2 Main mRNA–miRNA regulation in HepG2 cells treated with 2.3 μg ml^{-1} Cd as 5.2 μg ml^{-1} Cd(II). The figure depicts events of p53 signalling pathway. Despite the high levels of p53, cell cycle block was not an hypothesis (p21, GADD45, 14.3.3.δ are down-regulated). However, genes involved in oxidative stress (GSTM1, GSTTA1 and cytochrome p450 family genes are up-regulated) which may causes apoptosis. mRNAs or miRNAs that changed their abundance in response to the treatment are showed in red (increase) or green (decrease). As a result, the specific step is unlocked (green light) or locked (red light) or could be unlocked (yellow light). DIANA-Tarbase database, DIANA-mirPath and KEGG were used to create this chart. Some icons were created with **https://BioRender.com**.

showed a set of biological processes, including not only the inflammatory response, but also, more evidently, management of oxidative stress (Fig. S4b and Table S11†).

In our previous work,[25,30] through a miRNome analysis in THP-1 cells exposed to 50 μg ml^{-1} of CdS QDs, a set of regulated miRNAs involved in autophagy were identified. Furthermore, Western blot analysis showed an increase of the LC3II protein and constant p62 levels, all data confirming the involvement of the autophagic process, an intracellular degradation and energy recycling mechanism.[47] Here, at transcriptomic level, most of the major mRNAs previously identified as involved in autophagy did not show significant variations in their expression levels (Fig. 3 and Table S2†). Possibly, after 24 h of exposure to CdS QDs, the THP-1 cells had already formed most of the autophagosomes and, therefore, the induction of the genes involved in the process was no longer maintained.

Looking at the analysis on mRNA and miRNA interactions, it could be hypothesized that the QDs, once inside THP-1 cells, determine the activation of the JAK/STAT and AKT signal cascades, resulting in the release of cytokines, including tumor necrosis factor α (TNFα), which is common to several inflammatory processes such as those consequent to exposure to environmental contaminants,[48] including nanoparticulates.[49] Indeed, since some of the physicochemical characteristics of nanomaterials and ultrafine particles (UFPs) can overlap,[50,51] such as size, exposure routes, and metal content, their toxicity mechanisms might also share common pathways and mechanisms. Here, the exposure to QDs is linked with an inflammatory response, as occurs following exposure to UFP.[49] For major details see S1.3.†

Most cytokines favor inflammation, while others are anti-inflammatory. In particular, TNFα is an early pro-inflammatory cytokine and its dysregulated expression has been found to lead to extensive damage and multiorgan failure.[52] Besides its role in the early inflammatory response, TNFα can trigger extrinsic apoptosis (see Fig. 3 and 4). It is involved in endothelial cell activation, leukocyte recruitment, and progression to systemic inflammatory response and its chronicization.[53]

Another important point is the possibility that the induction of cytokines, including TNFα, is modulated by the QDs protein corona.[54] When immune cells encounter QDs,

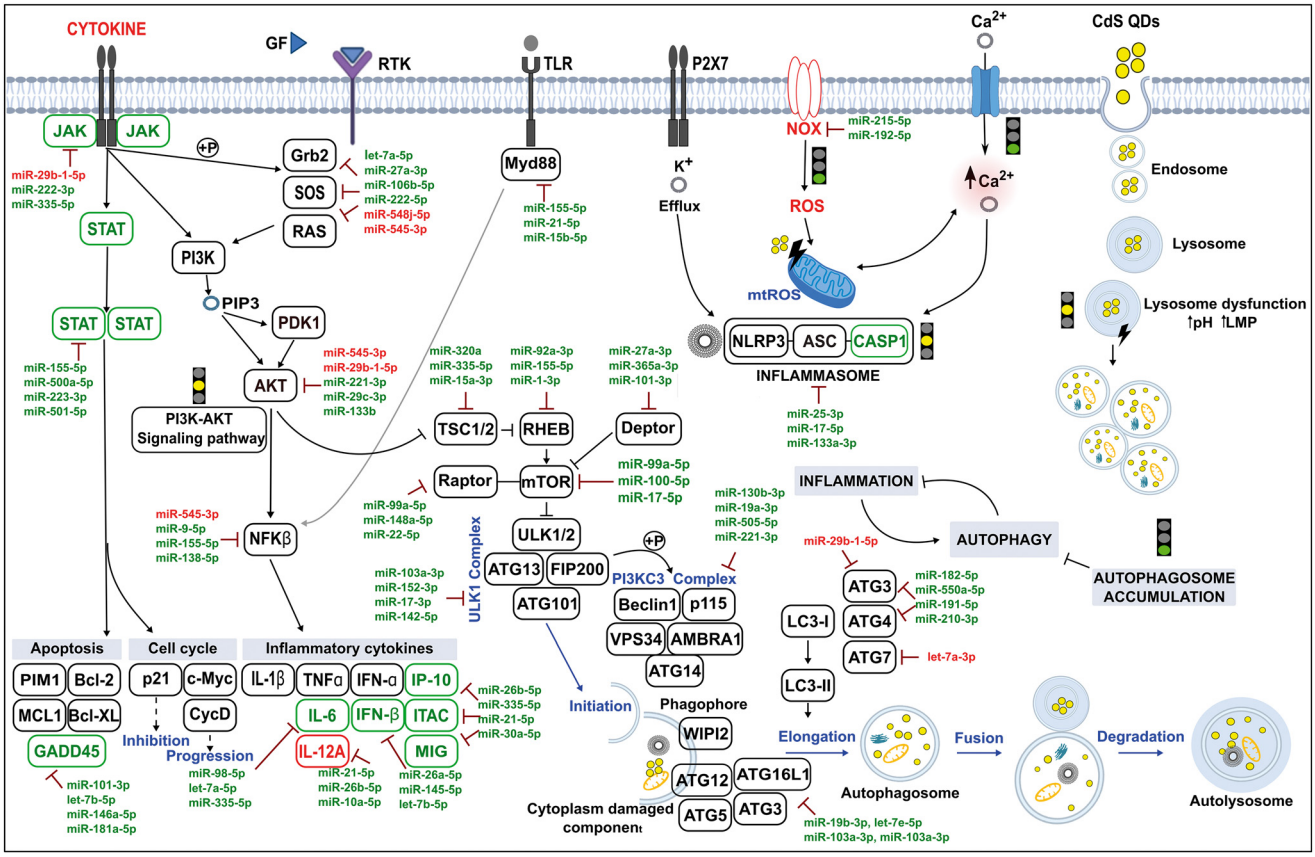

Fig. 3 Main mRNA–miRNA regulation in THP-1 cells treated with 39 μg ml^{-1} Cd as 50 μg ml^{-1} CdS QDs. The figure depicts events of principal pathways activated. The treatment determines the activation of the JAK/STAT and AKT signal cascades, resulting in the release of cytokines. Moreover the induction of NOXO1 and DUOX1, involved in oxidative stress, plays an important role in the autophagy triggered by QDs. mRNAs or miRNAs which changed their abundance in response to the treatment are showed in red (increase) or green (decrease). As a result, the specific step is unlocked (green light) or could be unlocked (yellow light). DIANA-Tarbase database, DIANA-mirPath and KEGG were used to create this chart. Some icons were created with **https://BioRender.com**.

they change the expression of surface markers and the secretion of cytokines.[55] In particular, the increase in TNFα levels was up to 200 times higher than the control after as early as 4 hours of exposure (Fig. S5†). Inflammation level drops significantly after 24 hours of exposure. This may explain why in the transcriptomic analysis, we do not observe an up-regulation of the genes involved in this pathway whereas the miRNAs involved in the regulation of these events are still present. In addition, as reported in Paesano *et al.*,[30] the mitochondrial energetic stress seems to be overcome because genes involved in the induction of mitophagy of damaged/dysfunctional mitochondria,[56] such as MFF, ULK1 and mitoAMPk, are not significantly transcribed after 24 h of exposure.

Nevertheless, the oxidative stress (as for example the induction of NOXO1 and DUOX1) caused by QD exposure plays an important role in the regulation of autophagy.[57] Oxidative stress, causing the accumulation of misfolded proteins and, consequently, endoplasmic reticulum (ER) stress and mitochondrial damage, may be responsible for autophagy trigger induced by nanomaterials.[58] Different studies have suggested that Cd-based QDs could induce autophagy, and oxidative stress-induced autophagy is considered a defense/survival mechanism against the cytotoxicity of QDs.[59,60] Additionally, the limited biotransformation of nanomaterials[61] in lysosomes can also directly induce ROS. Lysosomes are indeed considered conventional targets in cytotoxicity and autophagy induced by nanoparticles.[57]

A prolonged exposure to CdS QDs causes an accumulation of nanoparticles in the lysosomes causing alkalinization and damage, leading to the block of autophagic flow. QDs are indeed isolated in the lysosomes and, therefore, the biotransformation occurs here through a degradative action.[62] Interestingly, autophagy can negatively regulate the activation of the inflammasome,[63] a mechanism reminiscent of SARS-CoV-2 infection.[64] Autophagy induction is a cellular response typical of cells exposed to many nanomaterials, where it showed a size dependence.[57,65,66] However, while playing a cytoprotective role under certain conditions, it may also act as a pathway to cell death when overinduced.[67]

The possibility of using QDs to define some of the cellular mechanisms in response to UFPs is even more interesting given the recent COVID-19 pandemic. Indeed, knowing the

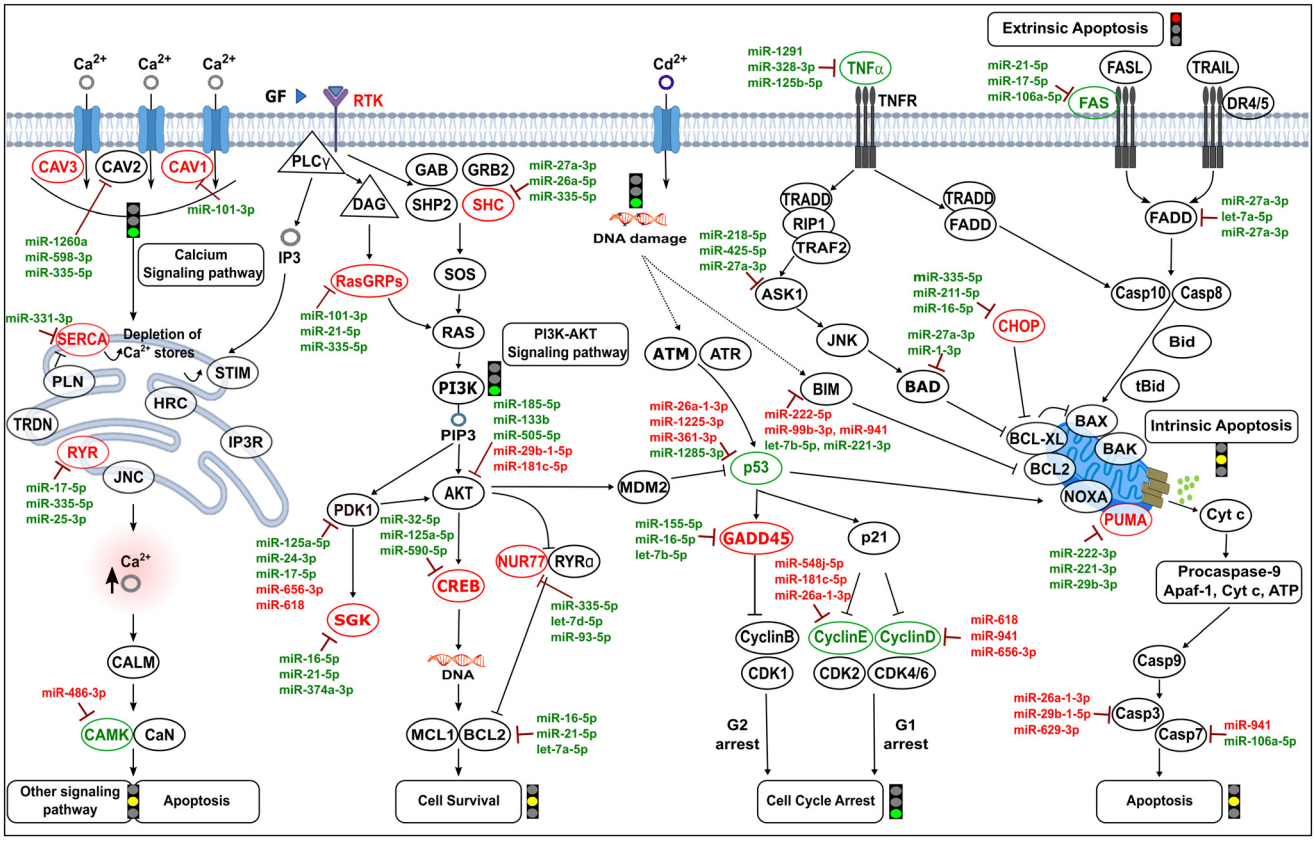

Fig. 4 Main mRNA–miRNA regulation in THP-1 cells treated with 5 μg ml^{-1} Cd as 11.4 μg ml^{-1} Cd(II). The figure depicts events relating calcium signalling pathway and apoptosis. Up-regulation of CAV1, CAV3, SERCA, RYR, RASGRPs suggests a significant increase in intracellular Ca^{2+} levels leading to the apoptotic process (PUMA is up-regulated). Furthermore, cell cycle blocking would also seem possible (GADD5 is up-regulated). mRNAs or miRNAs that changed their abundance in response to the treatment are showed in red (increase) or green (decrease). As a result, the specific step is unlocked (green light) or locked (red light) or could be unlocked (yellow light). DIANA-Tarbase database, DIANA-mirPath and KEGG were used to create this chart. Some icons were created with **https://BioRender.com**.

role that environmental factors including environmental pollution and, in particular, UFPs play in influenza virus outbreaks, these factors were considered as a potential key factor in the spread and mortality of COVID-19.[68,69] An interesting possibility is illustrated in Fig. 5 in which the mechanisms elicited by the nanomaterials were paralleled with those triggered by microparticulate[70] or by the infection of SARS-CoV-2.[64] There could be some common elements between them: activation of the MAPK cascade, synthesis of cytokines, and induction of the inflammatory response.[70] Thus, these results provide an interesting example of biotic and abiotic stress converging at the level of response mechanisms, which, under specific circumstances, may potentiate reciprocally (S1.3†). Indeed, Mescoli *et al.*[70] assumed that micro or nanoparticulates play a role as a booster of COVID-19 rather than as a carrier of SARS-CoV2, interplaying at the molecular level and amplifying the immune-mediated response.

Cellular response to Cd(II) in THP-1 cells have different outcomes than the response to CdS QDs (Fig. 4). Under this condition, apoptosis appears to be the most likely outcome. Exposure to Cd(II) causes a significant increase in intracellular calcium in THP-1, similar to that seen for HepG2 cells but more intense. In fact, high levels of mRNA, such as PUMA (p53 upregulated modulator of apoptosis), involved in the apoptotic process, are already expressed, as well as miRNAs that regulate it (Fig. 4), which are mainly down-regulated. Similarly, the GADD45 gene is over expressed and the miRNAs that regulate it are down-regulated. Moreover, as shown in the Fig. 4, the levels of mRNAs and of the correlated miRNAs appear to cause a cell cycle arrest following exposure to Cd(II).

Experimental

Preparation of CdS QDs

The CdS QDs were manufactured at IMEM-CNR (Parma, Italy). The same batch, whose synthesis information and characterization are given in Paesano *et al.*,[30] Pagano *et al.*[71] and summarized in ESI,† was used. In particular, their crystalline structure is that of wurtzite, with a mean static diameter of 2–5 nm, and approximately 78% of Cd. The average particle size (d_h) and zeta (ζ) potential of the CdS QDs (100 mg l^{-1}) were determined in ddH_2O and in medium on a Zetasizer Nano

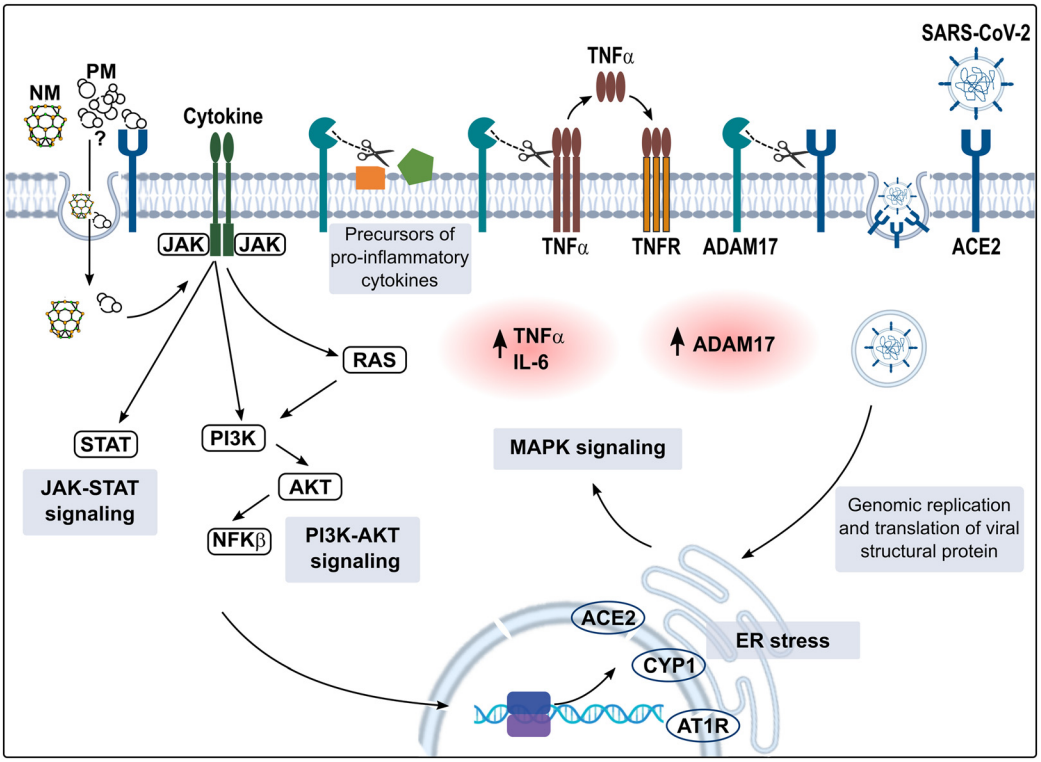

Fig. 5 Principal shared points of PM (microparticulate), NM (nanomaterial) and SARS-CoV2-mediated inflammation. After the binding of (S) SARS-spike to ACE2, the protein undergoes a proteolytic cleavage, and the virus enters the host cell. The attachment of the S protein to ACE2 triggers ADAM17 activation. The increased ADAM17 level exacerbates the imbalance of RAS in a looping feedback and increases inflammation, as indicated by increase of TNFα and IL-6.[80–83] After PMs and NMs enter the cell, they stimulate the production of inflammatory molecules, including IL-6 and TNFα. The production of cytokines is linked to the prolonged activation of the MAPK (ERK 1/2, p38)-STAT3 pathway, as confirmed by the increased levels of p-ERK1/2 and p-STAT3 levels.[55,84–86] ER stress is caused by PM, NM and viral infections.[11,58,87,88] Some icons were created with **https://BioRender.com**.

Series ZS90 (Malvern Instruments, Malvern, UK). The dispersion of QDs in the medium give rise to a suspension. The dissolution of QDs in the suspension was increased by different type of treatments as vortexing and sonication. The latter produces the more evident results because it also reduces the agglomeration of the nanoparticles of QDs.[72] In particular, according to Pagano *et al.*,[71] the release of Cd in water by CdS QDs in 7 days is 0.5%, which means that in 24 hours, the time of the experiment, there is almost no release of Cd by the CdS QDs. These results were also shown in Paesano *et al.*[30] and confirmed here.

The CdS QDs were suspended in Milli-Q water at a concentration of 100 μg ml^{-1}, pulsed probe sonication (Transsonic T460 device, Elma, Singen, Germany), at 35 kHz for 30 min, was used to minimize agglomeration. For cell treatment, the stock particle suspension was vortexed and sonicated for 30 min (Transsonic T460 device, Elma), and then diluted as appropriate into complete culture medium.

Cell culture and CdS QDs treatment

Human cell lines HepG2 and THP-1 were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 μg ml^{-1} streptomycin, 100 U ml^{-1} penicillin, 4 mM glutamine; for THP-1 cells, the glutamine concentration was reduced to 2 mM. Both HepG2 and THP-1 cell line were originally provided by the Cell Bank of the Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia-Romagna (Brescia, Italy). Cells were cultured in 10 cm Petri dishes under a humidified atmosphere in the presence of 5% CO_2 at a density of 3×10^6 cells per dish. Prior to treatment, THP-1 cells were differentiated into macrophages through an incubation with 0.1 μM of phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich, St. Louis, MO, USA, CAS number 16561-29-8) for 3 days.

The medium was then replaced with fresh medium containing either CdS QDs or $CdSO_4$ 8/3-hydrate (Sigma Aldrich, St. Louis, MO, USA, CAS number 7790-84-3), here abbreviated as Cd(II). Cells were treated with Cd as CdS QDs or Cd(II) for 24 h (Table S3†) and treatments were carried out in triplicate (biological replicates). Each replicate was measured three times (technical replicates).

The treatment doses were chosen on the basis of an assay of cell viability corresponding to IC_{20} (ref. 30) (see also Table S3†).

RNA isolation and mRNA expression profiling

Cells were exposed to a sub-toxic dose of CdS QDs and an equivalent dose of cadmium as Cd(II). In particular, HepG2

cells were exposed to 2.3 μg ml^{-1} Cd as 3 μg ml^{-1} of CdS QDs and 5.2 μg ml^{-1} of Cd(II); THP-1 cells were exposed to 5 μg ml^{-1} Cd as 6.4 μg ml^{-1} of CdS QDs and 11.4 μg ml^{-1} of Cd(II) and to 39 μg ml^{-1} Cd as 50 μg ml^{-1} of CdS QDs and 89 μg ml^{-1} of Cd(II) (Table S3†). For THP-1, the results reported are referred to 50 μg ml^{-1} of CdS QDs and 11.4 μg ml^{-1} of Cd(II) treatments. The treatment with 6.4 μg ml^{-1} of CdS QDs produced almost no effect on mRNAs profiling and that with 89 μg ml^{-1} of Cd(II) affected the cell viability, severely.[30]

These doses were established following Paesano *et al.*,[16,73] in which inhibitory and sub-inhibitory concentrations were determined.

Total RNA from treated and control cells was then extracted using a mirVANA™ miRNA Isolation kit (Ambion, Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Each RNA sample was monitored by gel electrophoresis for integrity and quantified spectrophotometrically.

mRNAs from each of the two cell lines, both treated and control, were used for RNAseq analysis. Protocol followed the manufacturer's instructions as developed at IGA Technology Services (Udine, Italy). In particular, the samples were sequenced on 75 bp single-end mode on NextSeq 500 (Illumina, San Diego, CA) producing on average 40 M of reads per sample. The CASAVA 1.8.2 version of the Illumina pipeline was used to process raw data for both format conversion and de-multiplexing. Cuffdiff tool was used to perform comparisons between the expression levels of genes and transcripts by calculating the FPKM (fragments per kilobase million) of each transcript.

Expression levels in treated cells were compared with those in the controls; fold change variations were calculated as the ratio between the averages of FPKM of the treated sample and FPKM of untreated sample in base 2 log scale. Genes showing fold change above or below 2 (cut-off chosen), with p-value < 0.05, were regarded as regulated (up or down) and were selected for further analysis.

Determination of TNFα

TNFα secretion in the culture media of THP-1 cells exposed to 50 μg ml^{-1} of CdS QDs was determined with the Human TNFα ELISA kit (cat. IK4185, Immunological Sciences, Rome, Italy) and following the manufacturer's instructions. More details are given in ESI† (S1.1.2).

Bioinformatics tools and statistic analysis

The RNAseq results were visualized using the CummeRbund package in the statistics environment R (**https://www.R-project.org/**).[74] The CummeRbund package is available from the Bioconductor website.

To obtain additional biological information from rough values of differentially expressed genes (DEGs), a comprehensive functional gene annotation was performed to improve the interpretation of the biological significance, using Gene Ontology database. PANTHER (**https://www.pantherdb.org/**) software was used to search for gene enrichment. This software use Fisher's exact test and false discovery rate (FDR) as calculated by the Benjamini–Hochberg procedure. All results shown are valid for p-value $<$ 0.001 and FDR < 0.05.

The preliminary analysis of genes targeted by differentially abundant miRNAs were identified using the DIANATarbase v.7 database (**https://diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index**).[75]

The multiMiR, an R package, was used to get a complete picture of miRNA–target interactions. It is a comprehensive collection of predicted and validated miRNA–target interactions and their associations with diseases and drugs.[76]

The degree of miRNA repression to a specific mRNA$_k$ was calculated with the following formula:

$$R_{\mathrm{mRNA}_k} = \left[\sum_{i=1}^{n} \left(\text{count miRNA}_i / n_{ij}\right)\right] / N_{\mathrm{Total}}$$

in which R_{mRNA_k} is the repression degree to the mRNA$_k$ by its targeted miRNAs; count miRNA$_i$ is the count of the ith miRNA; i is the number of miRNAs target to the mRNA$_k$, I = 1, 2, … n; n_{ij} is the number of mRNAs targeted by the miRNA$_i$; N_{total} is the number of all miRNAs detected in a small RNA sample.[77]

The Z-test assay was performed to investigate the differences in the repression of target mRNAs by miRNAs in nanomaterial-treated cells. p_0 was calculated by the following formula, which was the estimate of the proportions if the null hypothesis was true.

$$p_{0k} = \frac{\left[R_{\mathrm{mRNA}_k} \cdot N_{\mathrm{Total}}\right]_{\mathrm{Treat.}} + \left[R_{\mathrm{mRNA}_k} \cdot N_{\mathrm{Total}}\right]_{\mathrm{CTR}}}{N_{\mathrm{Total}_{\mathrm{Treat.}}} + N_{\mathrm{Total}_{\mathrm{CTR}}}}$$

$$Z_k = \frac{R_{\mathrm{mRNA}_{k\,\mathrm{Treat.}}} - R_{\mathrm{mRNA}_{k\,\mathrm{CTR}}}}{\sqrt{p_{0k}\left(1-p_{0k}\right)/N_{\mathrm{Total\,Treat.}} + p_{0k}\left(1-p_{0k}\right)/N_{\mathrm{Total\,CTR}}}}$$

When the Z-value was <-1.96 or >1.96, the null hypothesis was rejected, which meant that there were significant differences between the repression of mRNAs in the nanomaterial-treated and the control cells.[77] The R software (**https://www.r-project.org/**) was used for principal component analysis (PCA).

For TNFα data, the software package IBM SPSS Statistics1 v.21 (Armonk, NY, USA) was used to analyse results. In one-way analysis of variance, the Tukey test was applied to order means differing significantly from one another. The significance threshold was set at $p < 0.05$.

Conclusions

Utilization of QDs has risen in the last ten years, leading to their increased presence in the environment along with concerns for their impact on human health.[5,78,79] This work describes the responses to CdS QDs in two of their main

target cell types, hepatocytes and macrophages, represented, respectively, by HepG2 and THP-1 cell lines. In a previous paper we have described the regulation by a large set (754) of human miRNAs in response either to CdS QDs or Cd(II). Here, we link these data (*in silico*) with the *in vitro* response in transcriptional regulation of mRNA, using the formulae previously described. The data showed some consistency with the previous *in silico* prediction but also some differences. We observed that QDs induced the activation of RAS and Ca^{2+} signalling pathways in HepG2 cells, leading to apoptosis, while in THP-1 cells CdS QDs caused a saturation of autophagosomes. In early exposure phase, QDs determined the activation of the JAK/STAT and AKT signal cascades in THP-1 cells, resulting in the release of cytokines and the triggering of inflammatory cascade. A more prolonged exposure to QDs determined instead the down-regulation of IL-6 gene, suggesting a general silencing of inflammatory genes because of the block of the autophagic process, given to the accumulation of QDs in the lysosomes.

The observed cellular response to QDs could provide information on the adverse effects of other micro- or nano-structured environmental pollutants (S1.4†). This represents an interesting possibility given the difficulty in performing *in vitro* toxicological studies with UFPs due to challenges related to sampling procedure and due to the heterogeneity of the material collected, specifically in relation to the large amount of UFPs particle mass necessary to perform accurate *in vitro* studies.

Author contributions

Laura Paesano: data curation, formal analysis, investigation, writing – original draft. Megi Vogli: formal analysis, investigation. Marta Marmiroli: writing – review & editing. Massimiliano G. Bianchi: investigation, writing – review. Ovidio Bussolati: writing – review. Andrea Zappettini: validation. Nelson Marmiroli: conceptualization, funding acquisition, supervision, writing – review & editing.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this paper.

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