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Chapter 17

Role of Phytochelatins in Redox Caused Stress in Plants and Animals

Miguel Angel Merlos Rodrigo, Naser A. Anjum, Zbynek Heger, Ondrej Zitka, Adam Vojtech, Eduarda Pereira and Rene Kizek

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Abstract

Varied environmental compartments (such as soil and water) potentially contaminated with different metals/metalloids can impact the health of both plants and animals/humans. Trace amounts of Cu, Mn, Mo, Ni and Zn are beneficial for higher plants, whereas, Cr, Cu, Co, Mn, Mo, Se, V and Zn are known as the micronutrient metal/metalloids for animals/humans. However, elevated levels of the metals/metalloids can cause severe toxic consequences in both plants and animals/humans. Common in plants and animals/humans, phytochelatins (PCs), the principal non-protein, S-rich, thiolate peptides, protect (through different mechanisms) cellular functions and metal/metalloid homeostasis by performing their chelation and/or detoxification. With the major aim of broadening the current knowledge on the subject, this chapter (a) overviews PCs' role and modulation separately in metal/metalloid-exposed plants and animals/humans; (b) discusses major methods for determination of PCs and bioassays for enzymes involved in PC synthesis; (c) evaluates the connection of PCs with bionanoparticles; and finally (d) highlights so far unexplored aspects in the present context.

Keywords: Phytochelatin, metal, glutathione, stress

1. Introduction

Anthropogenic activities have caused the release of a wide range of hazardous metals/metalloids (hereafter termed as 'metal/s') into the environment. In particular, increasing emissions of metals such as Cd, Hg and As into the environment pose an acute problem for all organisms. Metals, unlike organic contaminants, are not degradable and remain persistent in soils [1–3]. Once taken up, these metals can bring severe toxic consequences in cells due to their chemical similarity to replace the metals necessary for cellular functions. Nevertheless,

metals at toxic levels have the capability to interact with several vital cellular biomolecules such as nuclear proteins and DNA, leading to excessive augmentation of reactive oxygen species (ROS) [4–6]. In addition, these metals generate ROS which in turn can cause neurotoxicity, hepatotoxicity and nephrotoxicity in humans and animals [7, 8]. Notably, higher plants, algae, certain yeasts and animals are equipped with a repertoire of mechanisms to counteract metal toxicity. The key elements of these are chelation of metals by forming phytochelatins (PCs) and related cysteine-rich polypeptides [9–11]. PCs are produced from glutamine, cysteine and glycine and the process is catalysed by PC synthases known as γ-glutamylcysteine (γ-Glu-Cys) dipeptidyl transpeptidases [12, 11]. PCs have been identified in a wide variety of plant species, microorganisms and invertebrates. They are structurally related to glutathione (GSH) and were presumed to be the products of a biosynthetic pathway. Numerous physiological, biochemical and genetic studies have confirmed that GSH is the substrate for PC biosynthesis [13, 14]. The general structure of PCs is (γ-Glu-Cys)n-Gly, with increasing repetitions of the dipeptide Glu-Cys, where *n* can range from 2 to 11 but is typically no more than 5 [15]. Except glycine, other amino acid residues can be found on the C-terminal end of (γ-Glu-Cys)*n* peptides. In Figure 1, we show the general structure of PC and the major steps involved in its synthesis from GSH through PC synthase in response to high concentrations of toxic metals. Originally thought to be plant-specific, PC and PC synthases have now been reported in a few fungal taxa, such as the yeast *Schizosaccharomyces* sp. and the mycorrhizal ascomycete *Tuber melanosporum* [16, 17] and invertebrates belonging to the nematodes, annelids or plathyhelminths [18, 19, 4, 1, 20, 17, 21–24].

In the light of recent literature, the PCs' role and modulation are overviewed separately in metal-exposed plants and animals/humans and major methods for the determination of PCs and the bioassays for enzymes involved in PC synthesis are discussed hereunder. Additionally, connection of PCs with bionanoparticles is evaluated, and finally, major aspects so far unexplored in the present context are briefly highlighted.

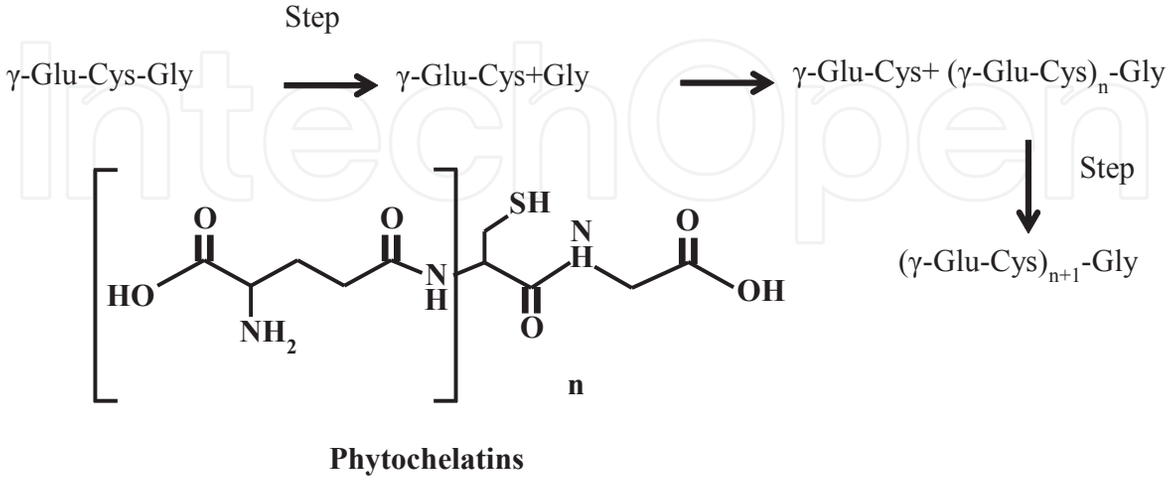

Figure 1. General structure of phytochelatins (PCs) and the major steps involved in its synthesis from glutathione (GSH) through a PC synthase in response to high concentrations of toxic metals.

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2. Phytochelatins in metal/metalloid-exposed plants

Contamination by metals can be considered as one of the most critical threats to soil and water resources as well as to human health [25, 26]. In fact, the contamination of soils with toxic metals has often resulted from human activities, especially those related to accelerated rate of industrialization, intensive agriculture and extensive mining. Metal belongs to group of non-biodegradable, persistent inorganic chemical having cytotoxic, genotoxic and mutagenic effects on humans or animals and plants through influencing and tainting food chains, soil, irrigation or potable water and aquifers [27, 28, 6]. Chelation and sequestration of metals by particular ligands are the major mechanisms employed by plants to deal with metal stress. The two best-characterized metal-binding ligands in plant cells are the PC and metallothioneins (MTs) [29–33, 6, 34].

Figure 2 shows the scheme of metal-detoxification by PCs in a plant cell. PC, which has a higher affinity for Cd, is formed by the polymerization of 2–11 γ-EC moieties via PC synthase. Several studies confirm that in plants, both GSH and PC synthesis are increased after exposure to Cd and other metals [12, 35–41]. In Figure 3, we show both general functions of the PC and a model of complex between Cd^{+2} ion and one molecule of PC.

Gonzalez-Mendoza et al. showed that PC synthase gene (in coordination with the expression of metallothionein gene) is present in *Avicennia germinans* leaves, and that their expression increases in response to metal exposure, which supports the hypothesis that PC synthase and metallothionein are part of the metal-tolerance mechanisms in this species. In addition, these authors found that *A. germinans* has the ability to express both genes (*AvMT2* and *AvPCS*) as a coordinated response mechanism to avoid the toxic effects caused by non-essential metals. However, for essential metals such as Cu^{2+}, the results showed that *AvPCS* was the most active gene involved in the regulation of this metal in the leaves [42]. Recent study showed that *Lunularia cruciata* compartmentalizes Cd^{+2} in the vacuoles of the photosynthetic parenchyma by means of a PC-mediated detoxification strategy, and possesses a PC synthase that is activated by Cd and homeostatic concentrations of Fe(II) and Zn. *Arabidopsis thaliana* PC synthase displays a higher and broader response to several metals (such as Cd, Fe(II), Zn, Cu, Hg, Pb, As(III)) than *L. cruciata* PC synthase [35].

Naturally hyperaccumulating plants do not overproduce PCs as a part of their mechanism against toxic metals. This appears to be an inducible rather than a constitutive mechanism, observed especially in metal non-tolerant plants [43]. Some reports have argued against the roles of PC in some metal-tolerant plants based on the effects of buthionine-S-sulphoximine and PCs/metal concentrations [44]. Several studies on plants overexpressing γ-glutamyl-cysteine synthetase or transgenic plants expressing bacterial γ-glutamyl-cysteine synthetase evaluated its effect on metal tolerance based on the assumption that higher levels of GSH and PCs will lead to more efficient metal sequestration [45]. *Bacopa monnieri*, a wetland macrophyte, is well known for its accumulation potential of metals and metal tolerance and thus is suitable for phytoremediation. Aquatic plants respond to metal stress by increasing the production of PC as well as other antioxidants. The accumulation potential of *B. monnieri* for various metals warrants its evaluation for metal tolerance and detoxification mechanism and for its suitability

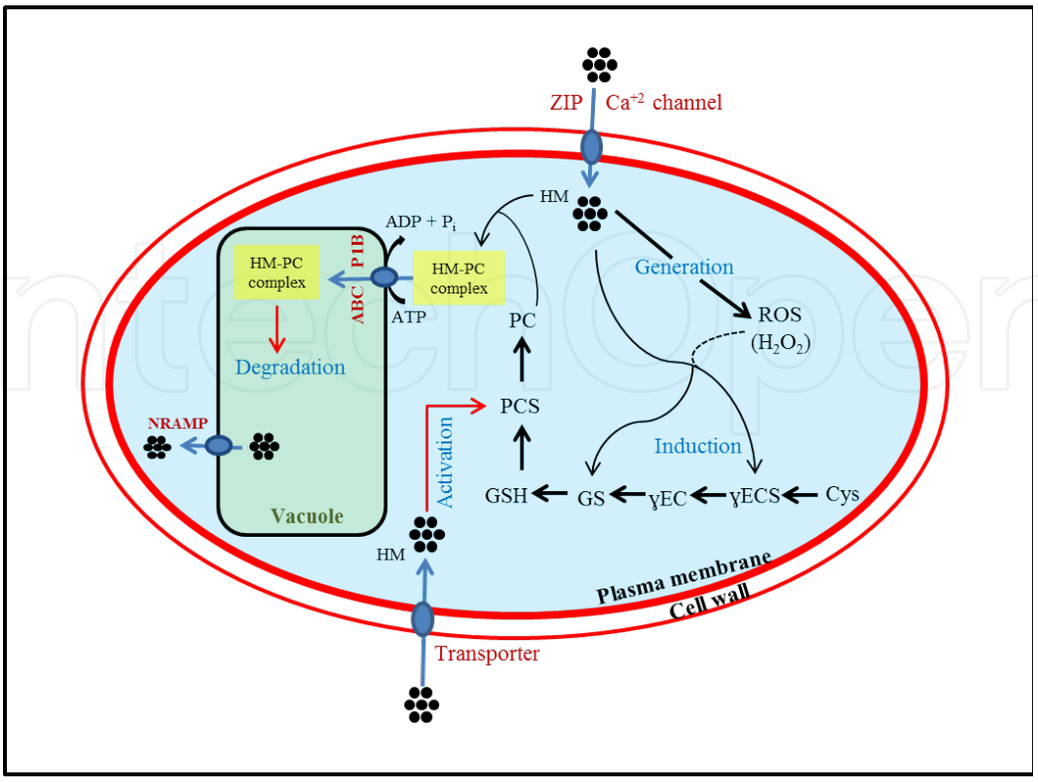

Figure 2. The scheme of heavy metal (HM) detoxification by phytochelatins (PC) in a plant cell. HM activates phytochelatin synthase (PCS) and the HM–PC complexes are established. These complexes are consequently transported through tonoplast to vacuole by ATP-binding-cassette and P1B-ATPase transporter (ABC-P1B). HM is chelated in the cytosol by ligands such as PC. Induction of PC synthesis by HM and a large flux of GSH is further achieved by increased activity of the GSH metabolic enzymes, γ-ECS and GS. It is possible that the enzyme activation is not directed through effects of HM but due to H2O2 produced as a result of HM-presence. Transport of HM through the plasma membrane (ZIP). Vacuolar transport of HM (NRAMP: natural resistance associated macrophage protein). Heavy metals are shown as black dots. Figure adapted and modified from [26].

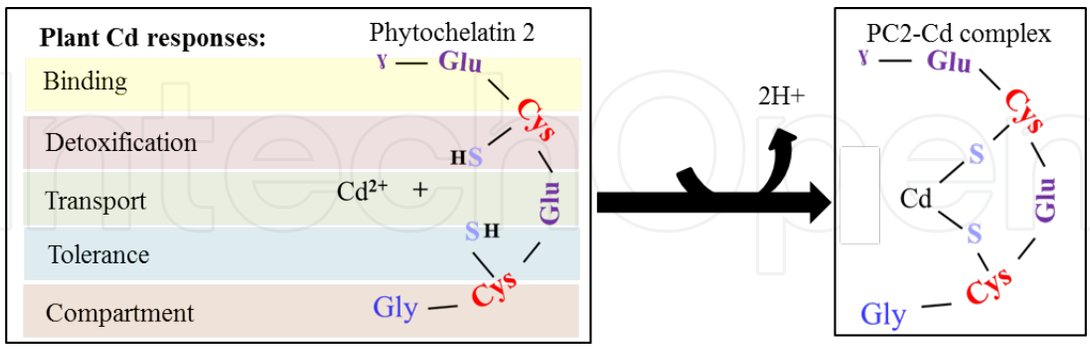

Figure 3. General functions of phytochelatins (PCs) and the model of complex between cadmium (Cd^{+2}) ion and one molecule of PC2. Cys, cysteine; Glu, glutamic acid; Gly, glycine; S, sulphur.

in phytoremediation [38]. In a study on *Arabidopsis thaliana* showed that Cd is immediately scavenged by thiols in root cells, in particular PC, at the expense of GSH. At the same time, a redox signal is suggested to be generated by a decreased GSH pool in combination with an

altered GSH:GSSG ratio in order to increase the antioxidant capacity [46]. Overexpression of PCs synthetase in Arabidopsis led to 20–100 times more biomass on 250 and 300 μM arsenate than in the wild type. Also, the accumulation of thiol-peptides was 10 times higher after the exposure to Cd and arsenic, compared to the wild type. Gamma-glutamyl cysteine, which is a substrate for PC synthesis, increased rapidly after arsenate or Cd-exposure. Overexpression of PC synthase gene can be useful for phytoremediation [47]. Additionally, legumes are also capable of synthesizing homo-PCs in response to metal stress [45]. Citrus plants were also reported to synthesize PC in response to metal intoxication [48]. In wheat (*Triticum aestivum*), PC–metal complexes have been reported to accumulate in the vacuole. Retention of Cd in the root cell vacuoles might influence the symplastic radial Cd transport to the xylem and further transport to the shoot, resulting in genotypic differences in grain Cd accumulation [49].

3. Phytochelatins in metal/metalloid-exposed animals

As mentioned also above, PC proteins have been broadly described and characterized in plants, yeasts, algae, fungi and bacteria [22]. However, PC synthase genes are also present in animal species from several phyla. PC synthesis appears not to be transcriptionally regulated in animals [50]. Nevertheless, originally thought to be found only in plants and yeast, PC synthase genes have since been found in species that span almost the whole animal tree of life. Notably, PC synthase genes are found in species from several other metazoan phyla, including Annelida, Cnidaria, Echinodermata, Chordata and Mollusca (both Gastropoda and Bivalvia classes) [51, 52].

Several phyla of the Metazoa contain one or more species harbouring PC synthase homologous sequences: the Cnidaria (*Hydra magnipapillata*), the Chordata (*Molgula tectiformis*, as well the model chordate *Ciona intestinalis*), the Echinodermata (*Strongylocentrotus purpuratus*), the Annelida (*Lumbricus rubellus*) and the Platyhelminthes (*Schistosoma japonicum* and *Schistosoma mansoni*) [53, 51]. Biochemical studies have also shown that these PC synthase genes are functional. The *Caenorhabditis elegans* PC synthase produces PC when it is expressed in an appropriate host, and knocking out the gene increases the sensitivity of *C. elegans* to Cd [54]. Several studies have since measured PC by direct biochemical analysis of *C. elegans* tissue extracts, and found that Cd exposure did indeed increase PC levels in *C. elegans*. PC2, PC3 and PC4 have all been found, with PC2 in the highest concentration [55, 20, 56]. Therefore, these studies concluded that PCs production can play a major role in protecting *C. elegans* against Cd toxicity. PC2 and PC3 were increased in autochthonous *Lumbricus rubellus* populations sampled from contaminated sites [50]. The yeast (for example, *S. pombe*) possesses an ATP-binding cassette (ABC) transporter, Hmt1, which was originally thought to play a possible role in translocation of PCs–metal complexes to the vacuole. However, while knocking out the *C. elegans* HMT-1 (CeHMT-1) increases the sensitivity to Cd; the increase is greater than could be explained by a lack of PC synthase alone [57]. It is important to say that MTs are another widely established metal-binding ligand and a key metal detoxification system in animals. Additionally, MTs have many other important biological functions

as well. Nevertheless, little is known about how MTs and PCs may complement each other for dealing with toxic metals [50].

The activation and function of PC synthase in animals came into light from studies on the nematode *C. elegans* [58], the flatworm *Schistosoma mansoni* [19, 59, 21], and Cionidae *Ciona intestinalis* [60]. The occurrence of PC synthase in animals suggests the occurrence, in these organisms, of a stress oxidative and metal detoxification system based on a class of molecules which was considered as the privilege of plants. The PC synthase gene has a wide phylogenetic distribution and can be found in species that cover almost all of the animal tree of life. But even though some members of particular taxonomic groups may contain PC synthase genes, there are also many species without these genes. Ron Elran et al. reported the regulation of GSH cycle genes in *Nematostella vectensis*, and an interesting finding was that PC synthase 1, which synthesizes the non-ribosomal formation of metal-binding PC, was upregulated after Hg and Cu treatments [15]. Phylogenetic analyses supported the hypothesis that PC synthase evolved independently in plants, cyanobacteria and green algae. Among the sequenced metazoan genomes, only a few contain a PC synthase gene. However, the reason for the scattered distribution of these genes remains unclear, considering that metazoans with PC synthase genes in their genomes do not share any physiological, behavioural or ecological features [60]. Just how (and if) PC in invertebrates complement the function of MTs remains to be elucidated, and the temporal, spatial and metal specificity of the two systems are still unknown [6].

4. Methods for the assays of phytochelatins and phytochelatin synthase enzyme

4.1. Determination of phytochelatins

We briefly discuss herein different methods for the detection and quantification of PC. Additionally, we are giving an overview of the methods used for determination of PC, comprising a broad range of electrochemical as well as spectrometric methods, which have been optimized and even hyphenated with different separation methods to detect PC. Recently, Wood et al. showed the analytical methodology for quantification of PC and their metal(loid) complexes [61]. The classical approach to the analysis of PC is reversed phase HPLC with post-column derivatization of the sulphydryl groups and spectrophotometric detection, but the detection is not specific to PC. The use of an analytical technique is able to detect compounds, specifically mass spectrometry. Independent studies showed a sensitive method for determining PCs by HPLC with fluorescence detection [62, 63]. A simple sensitive method for the identification, sequencing and quantitative determination of PCs in plants by electrospray tandem mass spectrometry (ESI MS-MS) was showed for different studies [64, 65]. Other study showed the combination of three processes for identification PC: (1) easy sample preparation including thiol reduction, (2) rapid and high-resolution separation using ultra-performance liquid chromatography (UPLC) and (3) specific and sensitive ESI-MS/MS detection using multi-reaction mode (MRM) transitions in alga's extract [66].

Nevertheless, in vitro formed Cd–PC2 complexes were characterized using ion exchange chromatography (IEC), flow injection analysis/high-performance liquid chromatography with CoulArray or Coulochem electrochemical detector and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry [67, 68]. Zitka et al. optimized high-performance liquid chromatography coupled with electrochemical detector for determination of PC2 [69]. Many studies showed the determination of cysteine, reduced and oxidized glutathione and PC in different species of plants using high-performance liquid chromatography with electrochemical detection [70, 71].

4.2. Bioassays for phytochelatin synthase activity

The methods for identification and quantification of PC synthase are multidisciplinary, among themselves, comprising a broad range of molecular biology, electrochemical and spectrometric methods. HPLC coupled with electrochemical detector has been suggested as a new tool for the determination of PC synthase activity. The optimized procedure was subsequently used for studying PC synthase activity in the tobacco BY-2 cells treated with different concentrations of Cd(II) ions and the results were in good agreement with Nakazawa et al. [72]. Other study in animals showed that HPLC-LC system coupled to a single quadrupole LC–MS equipped with ESI was a sensitive method for PC synthase activity [22]. A highly sensitive assay for PC synthase activity was devised, where, the dequenching of Cu(I)-bathocuproinedisulphonate complexes was used in the detection system of a reversed-phase high-performance liquid chromatography. The present assay method is a sensitive tool that can be used to investigate this issue and would allow for the determination of PC synthase activity using 10–100-fold less protein [73]. Electrochemical methods such as differential pulse voltammetry and high-performance liquid chromatography with electrochemical detection were used for determination of Pt(IV) content, GSH levels, PC synthase activity in maize (*Zea mays*) and pea (*Pisum sativum*) plants treated with various doses of Pt(IV) [74].

Other methods required for the identification and characterization of PC synthase are, for example, the novel technology of molecular biology. Xu et al. showed a study that represents the first transcriptome-based analysis of miRNAs and their targets responsive to Cd stress in radish (*Raphanus sativus*) roots. Furthermore, a few target transcripts including PC synthase 1 (PCS1), iron transporter protein and ABC transporter protein were involved in plant response to Cd stress [75]. In 2009, Amaro et al. reported the identification and characterization of a cDNA encoding a PC synthase homologous sequence from the ciliated protozoan *T. thermophila*, the first to be described in ciliates. A quantitative real-time PCR (qRT-PCR) expression analysis of PC synthase has been carried out under different metal stress conditions. Several experimental evidences suggest that this enzyme is biosynthetically inactive in PC formation, which makes it the first pseudo-PC synthase to be described in eukaryotes [76].

5. Phytochelatins in connection with bionanoparticles

The connection of nanoparticles and PC has two faces: on one hand, the biosynthesis of nanoparticles and on the other hand, the protection of stress caused by the damage of any

harmful nanoparticles. An *in vitro* study showed the enzyme-mediated synthesis of CdS nanocrystals by immobilized PC synthase, which converts GSH into the metal-binding peptide PC. Formation of CdS nanocrystals were observed upon the addition of CdCl2 and Na2S with PC as the capping agent [77]. This study is expected to help in designing a rational enzymatic strategy for the synthesis of nanoparticles of different chemical compositions, shapes and sizes. Also, an enzymatic synthesis route to peptide-capped gold nanoparticles was developed. Gold nanoparticles were synthesized using alpha-NADPH-dependent sulphite reductase and PC in vitro [78]. In Figure 4, we show the general structure of nanocrystal with cross-linked, PC-like coating (Figure modified from [79]). The microbiological production of inorganic nanoparticles is an interesting and promising alternative to the known physical and chemical production methods. Extensive studies revealed the potential of bacteria, actinomycetes, algae, yeasts and fungi for biosynthesis of nanoparticles [80]. Few studies have discussed the possible synthesis of nanoparticles by algae. Particularly, *Phaeodactylum tricornutum* exposed to Cd, forms Cd–PC complexes, where sulphide ions (S^{2-}) can be incorporated to stabilize PC-coated CdS nanocrystallites [81, 82]. Metal is immobilized by an intracellular detoxification mechanism. Krumov et al. showed that Cd is associated to a protein fraction between 25 and 67 kDa which correspond to the theoretical molecular weight of CdS nanoparticles of 35 kDa coated with PC by size exclusion chromatography [83]. However, contingent to their types and concentrations, any nanoparticles can pose a risk to human health and to the environment [84]. Zinc oxide nanoparticles (ZnONPs) are used in large quantities by the cosmetic, food and textile industries. The harmful effects of ZnONPs are driven by their physicochemical properties and the resulting physical damage caused by the aggregation and agglomeration of nanoparticles. PC synthase may confer protection against ZnONPs-induced toxicity in *Caenorhabditis elegans* [24]. Effect of magnetic nanoparticles on tobacco BY-2 cell suspension culture showed induced PC biosynthesis. These trends were observable for almost all monitored PCs: PC2, PC3 and PC5 [85].

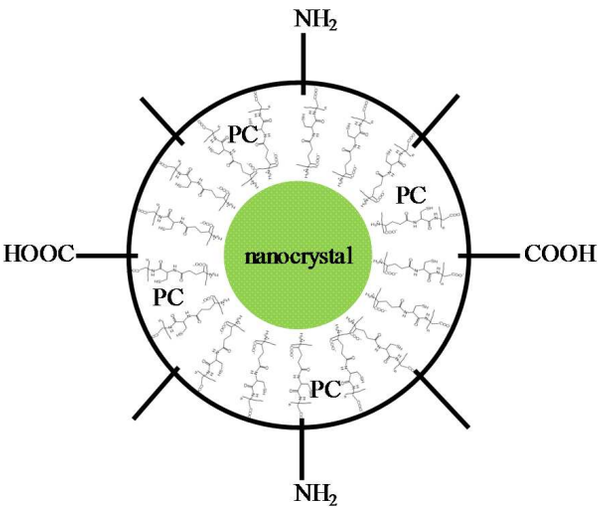

Figure 4. Nanocrystal with crosslinked, phytochelatin (PC)-like coating, an effective strategy to make QDs as small with a crosslinked peptide sheath by mimicking PC-coated heavy metal nanoclusters. Figure adapted and modified from [79].

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6. Conclusions

The concept of phytoremediation of contaminated soils has been increasingly supported by research in recent years. The identification of PC synthase genes from plants and other organisms is a significant breakthrough that will lead to a better understanding of the regulation of a critical step in PC biosynthesis. Many studies showed the mechanisms of chelation of metals–PC in plants in recent years. Chelation and sequestration of metals by particular ligands are also mechanisms used by plants to deal with metal stress. The two best-characterized metal-binding ligands in plant cells are the PCs and MTs. While the role played by PC synthase enzymes and PCs in animals still remains to be fully explored, there is increasing evidence that PC synthase genes are likely to be found in many important animal groups and that PCs may well turn out to be important players in metal ion detoxification in many of these species. It will be of interest in the future to see whether different animal species coordinate PC and MT responses to potentially toxic elements and if this is different for different metal ions.

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