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Application of *Solanum lycopersicum* (tomato) hairy roots for production of passivated CdS nanocrystals with quantum dot properties

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ABSTRACT

Semiconductor quantum dot particles have a wide range of applications in medicine, bioassays, computing and photovoltaics. Biological synthesis is an attractive approach for mass production of quantum dots as cells have the capacity to passivate the particles with organic ligands. In this work, hairy roots of *Solanum lycopersicum* (tomato) were used to produce CdS nanoparticles with quantum dot properties. Treatment of the roots with 100 µM Cd during the mid-growth phase of batch culture elicited cellular responses for Cd detoxification without affecting root growth. A combination of freeze-drying and freeze-thawing of the roots was used to extract Cd from the biomass; anion-exchange chromatography was then applied to selectively remove metal–phytochelatin complexes. Size-fractionation using gel filtration allowed the recovery of phytochelatin-capped Cd- and inorganic sulphide-containing nanoparticles displaying the size and size-dependent optical/electronic properties of CdS quantum dots. At 4–10 nm in diameter, these particles fluoresced at wavelengths corresponding to blue–violet on the colour spectrum and exhibited a high level of photostability with prolonged excitation. Whereas 69% of the Cd extracted from the roots was associated with phytochelatin peptides, the maximum yield of CdS nanocrystals with quantum dot properties was 1.4% of the total Cd taken up into the biomass. This work demonstrates a new culture-based approach for the biosynthesis of metallo-organic semiconductor quantum dots using hairy roots.

1. INTRODUCTION

Plants employ multiple defence mechanisms to protect against and neutralise the toxic effects of heavy metals. Uptake of excessive levels of heavy metals into the cytoplasm may be prevented by binding to the cell walls; detoxification also occurs at the molecular level through the synthesis of intracellular thiol-containing compounds such as metallothioneins, glutathione and phytochelatins. Complexation with these organic molecules reduces the cytotoxicity of metal ions and allows metal transport and storage in vacuoles. Phytochelatin peptides are widespread in the plant kingdom [1] and are comprised of three amino acids, Glu, Cys and Gly, arranged as $(\gamma\text{Glu-Cys})_n$-Gly with *n* ranging between 2 and 11 [2]. Phytochelatin synthesis in plants is strongly upregulated in the presence of metal ions such as Cd [1,2].

The metal-binding capacity of phytochelatins and related peptides has been exploited to enhance the chemical synthesis and photophysical properties of semiconductor quantum dots [3,4]. Quantum dot nanocrystals of materials such as CdS and CdSe have a wide range of potential applications in medical diagnostics, drug delivery, biological labelling, quantum computing and photovoltaics [5–9]. The diameter of nanocrystals displaying quantum dot properties is generally within the range 2–15 nm [10]. As the size of quantum dots is reduced within this range, the energy required to promote electrons from the valence to the conduction band, and the energy emitted as electrons return to the valence band after excitation, are increased, so that the UV-absorbance and fluorescence emission spectra, respectively, are blue-shifted compared with larger crystals [11]. Therefore, by changing the size of the particles, the optical properties of the crystals can be tuned to optimise their fluorescence emissions.

A wide range of chemical and organo-metallic methods has been used to synthesise quantum dots [10]. Capping quantum dots with biological compounds such as phytochelatins provides important benefits such as improved nanoparticle stability, monodispersion, water-solubility and biofunctionality [3,4]. Because phytochelatins are expressed constitutively in plants, and as plant cells intrinsically provide the space confinement conditions needed to limit

the size of developing nanocrystals, plant systems are a potential means for mass production of phytochelatin-capped quantum dots. Biological synthesis of CdS nanoparticles with quantum dot properties has been achieved previously using certain species of yeast, bacteria and algae [12–14]. For this to be successful in plants, sulphur must be incorporated into the Cd–phytochelatin complexes that are formed as part of the normal plant Cd detoxification response. Production of sulphide-containing Cd complexes of various size has been found to occur in a number of plant species [15–18]. However, demonstration of size-dependent semiconductor properties for plant-produced CdS nanoparticles has not yet been reported.

The aim of this work is to investigate the use of plant tissue culture for production of CdS quantum dots. *In vitro* plant cultures are preferable to field-grown plants for this purpose, as application of Cd to soil has serious and undesirable environmental effects. Because hairy roots display enhanced genotypic and phenotypic stability compared with dedifferentiated plant cells such as callus and suspension cultures [19], hairy roots of tomato produced by infection of plants with *Agrobacterium rhizogenes* were applied in this study. Here we report the response of tomato hairy roots to Cd treatment, methods for nanoparticle extraction and purification from root tissue, and the chemical, optical and size properties of the CdS nanocrystals produced.

2. MATERIALS AND METHODS

2.1 Initiation and maintenance of hairy roots

Seedlings of *Solanum lycopersicum* (tomato) cv Grosse Lisse were infected with *Agrobacterium rhizogenes* strains 15834 and A4. Hairy roots formed on solid Murashige and Skoog (MS) medium in the dark at 25°C were excised and transferred to Gamborg's B5 liquid medium containing 200 mg L^{-1} Claforan (cefotaxime) antibiotic and 3% w/v sucrose at pH 5.8. Antibiotic was removed from the medium after 3–4 subcultures. The cultures were maintained in 250-mL shake flasks in the dark at 25°C on orbital shakers operated at 50 rpm.

2.2 Culture experiments

Cultures were inoculated using 0.2 g fresh weight of roots from 21-day-old cultures and 50 mL of B5 medium in 250-mL shake flasks. $CdSO_4$ was added 5, 14 or 25 days after inoculation to give a concentration of 100 µM. Cd was not added to control cultures. To test the effect of medium replacement at the time of Cd addition, Cd was added to flasks 14 days after inoculation by replacing the medium with fresh B5 medium containing 100 µM $CdSO_4$. Control cultures with medium replacement at Day 14 without Cd were also carried out. Triplicate cultures were harvested periodically for measurement of biomass dry weight and Cd content.

To examine the effect of the duration of Cd treatment, Cd was added to root cultures with medium replacement at Day 14 and triplicate cultures were harvested 4, 7 and 9 days later for measurement of Cd and inorganic sulphide contents. Roots exposed to Cd for 9 days were used for nanoparticle purification.

2.3 Extraction of root biomass

Four different methods were tested for extraction of CdS nanoparticles from hairy roots.

2.3.1 Chopping

Roots were removed from the culture flasks and chopped repeatedly against the wall of a Falcon tube for 1–2 min using a single-sided razor blade with a long handle. When the roots were finely minced, extraction buffer containing 50 mM HEPES and 0.2% Tween 20 at pH 8.2 was added together with 1.5 mL per 100 mL of protease inhibitor cocktail for plant extracts (Sigma-Aldrich, USA). After vortexing, the mixture was centrifuged at 11,000 *g* for 20 min at 4°C. The supernatant was collected and the remaining solid material was subjected to further chopping and extraction. In total, four chopping and extraction cycles were applied. The supernatants from all extraction cycles were pooled and filtered using a 0.45-µm filter.

2.3.2 Freeze–Thawing

Roots were removed from culture flasks, frozen in Falcon tubes at –20°C, then thawed on ice for 2–3 h. Ice-cold extraction buffer was added with vortexing. Another volume of extraction buffer was added and the roots were frozen again at –20°C overnight, followed by thawing. The samples were vortexed and then centrifuged at 11,000 *g* for 20 min at 4°C. The supernatant was collected and another two cycles of freeze–thawing were applied to the remaining solids. After a total of four freeze–thaw cycles, the roots were centrifuged and the supernatants pooled and filtered.

2.3.3 Combination of Chopping and Freeze–Thawing

Roots were treated using one freeze–thaw cycle as described in Section 2.3.2. The thawed roots were then chopped as described in Section 2.3.1. After addition of extraction buffer, the samples were re-frozen overnight. After thawing, the roots were chopped again then extracted using extraction buffer with vortexing and centrifugation. The roots remaining in the tubes after removal of the supernatant were chopped again, extraction buffer was added, and the samples were vortexed and re-frozen. In total, four cycles of chopping and freeze–thawing were applied. The supernatants from all extraction cycles were pooled and filtered.

2.3.4 Combination of Freeze-Drying and Freeze–Thawing

Roots were removed from culture flasks, frozen at –70°C, and then freeze-dried overnight. The dried biomass was ground using a mortar and pestle, placed in a Falcon tube with extraction buffer, and vortexed. The mixture was frozen overnight at –20°C then thawed as described in Section 2.3.2. Four cycles of freeze–thawing were applied. The supernatants from all extraction cycles were pooled and filtered.

2.4 Purification of CdS nanoparticles

The root extracts were purified to isolate CdS nanoparticles. Cd–phytochelatin complexes are polyanionic and strongly negatively charged, as all of their amino acid carboxyl groups are oriented towards the external surface [18]. Anion-exchange chromatography was therefore applied to selectively remove Cd–phytochelatin complexes. The recovered samples were then size-fractionated using gel filtration.

Root extract was injected into four columns of HiTrap IEX Sepharose Fast Flow packing (Amersham, Sweden) in series. The columns were washed with buffer (50 mM HEPES, pH 8.2, with 0.2% v/v Tween 20) and eluted using a 0–1.5 M KCl gradient. Fractions collected as 2-mL volumes were analysed for Cd and inorganic sulphide contents and optical properties.

Ion-exchange chromatography fractions exhibiting properties consistent with the presence of CdS quantum dots were pooled, concentrated by freeze-drying, and size-fractionated by gel filtration using Sephadex G-50 fine XK 26/40 packing (Amersham). Fractions were collected as 1-mL volumes for measurement of Cd and inorganic sulphide contents and optical properties.

2.5 Analyses

2.5.1 Biomass

Hairy roots were filtered on Whatman No. 1 filter paper. Biomass dry weight was obtained by drying the roots in an oven at 50°C to constant weight.

2.5.2 Cd and inorganic sulphide

Samples of dried root, biomass extracts and chromatography fractions were digested in 70% w/w nitric acid at 80°C and analysed for Cd using atomic absorption spectrophotometry (AAS).

Inorganic or acid-labile sulphide contents in biomass extracts and chromatography fractions were measured based on the method of King and Morris [20]. Samples were transferred to 2-mL Eppendorf tubes and made up to 0.7 mL with Milli-Q water. NaOH (6% w/v in water, 0.1 mL) and zinc acetate (2.6% w/v in water, 0.5 mL) were added and the tubes promptly

recapped. After vortexing for 1 min, 0.25 mL of *N*-*N*-dimethyl-*p*-phenylenediamine monohydrate (0.1% w/v in 0.6 N HCl) was added. The mixture was inverted gently, 0.1 mL of iron III chloride (0.0118 M in 5 N HCl) was added, and the reactants were mixed by vortexing for 1 min. The tubes were incubated at room temperature for 30 min and then centrifuged to precipitate proteins. After addition of 0.85 mL of Milli-Q water, the absorbance was measured at 750 nm and compared with the results from reference solutions.

The sulphide concentration in reference solutions was determined using the iodometric method [21]. A 5-mL aliquot of standard sulphide solution was diluted to 100 mL using 0.25 M NaOH. Glacial acetic acid (1.5 mL in 15 mL of Milli-Q water) was added, followed by 5 mL of 0.1 N iodine acidified with 8 mL of 6 N HCl. A drop of starch indicator solution was added to the mixture, which was then back-titrated against 0.1 N sodium thiosulphate. The difference between the original volume of iodine added (5 mL) and the volume of unreacted iodine titrated with sodium thiosulphate was determined; the quantity of sulphide reacted with the iodine was then calculated as 1 mL of 0.1 N iodine reacts with 1.6 mg of sulphide.

2.5.3 Absorbance and fluorescence

The absorbance of root extracts and chromatography fractions was measured using a Nanodrop 1000 scanning UV–visible spectrophotometer. Fluorescence spectra were obtained using a Perkin-Elmer LS 50B fluorescence spectrometer operated over a range of excitation and emission wavelengths. The photostability of fluorescence emissions was examined using time-drive tests. In these experiments, the fluorescence spectrometer was set at a fixed excitation wavelength and the sample fluorescence was monitored at a fixed emission wavelength over a 2-h period.

2.6 Estimation of nanoparticle size from fluorescence emission characteristics

As the size of particles approaches the Bohr radius of the bulk exciton, quantum size-related effects may result in a blue shift in the band-gap energy of the particles [22]. CdS has a Bohr

exciton radius of ~3 nm [23], and quantum size effects have been observed in CdS nanocrystals of diameter up to 5–6 nm [24]. The shift in band-gap energy for size-fractionated CdS nanoparticles in hairy root gel filtration fractions was estimated from the wavelength of their peak fluorescence emissions. The Planck–Einstein equation relates the energy of light (E) to its wave frequency (v):

$$E = hv$$

where h is Planck's constant (= 4.13567×10^{-15} eV s). This relationship expressed in terms of wavelength (λ) is:

$$E = \frac{hc}{\lambda}$$

where c is the speed of light (= 2.998×10^{17} nm s^{-1}). The shift in band-gap energy ΔE_g for the nanoparticles was determined as the difference between E and the band-gap energy for bulk CdS, 2.42 eV [25].

The approximate sizes of CdS nanocrystals produced by hairy roots were estimated from the measured ΔE_g values using the parabolic effective mass approximation. This simple approximation assumes that the particles are spherical and that Coulomb interactions are negligible relative to confinement effects. The effective mass approximation applies in the strong confinement regime and has been shown to be valid for particles with radii up to twice the Bohr exciton radius [22,26]: for CdS, this includes nanoparticles up to ~6 nm in radius. According to the effective mass approximation, the relationship between the shift in band-gap energy ΔE_g and particle size is:

$$\Delta E_g = \frac{h^2}{8MR^2}$$

where R is the particle radius and M is the effective mass of the electron–hole pair defined as:

$$M = \mu = \frac{1}{\frac{1}{m_e} + \frac{1}{m_h}}$$

where μ is the reduced mass, m_e is the effective mass of the electron and m_h is the effective mass of the hole. For CdS, m_e = 0.18 electron mass units and m_h = 0.53 electron mass units [27]. Discrepancies have been found between the particle sizes estimated using the effective mass approximation and experimentally measured values for CdS particles of radius less than about 2 nm [28]. These limitations [24], together with the difficulty of determining peak emission wavelengths from broad experimental emission spectra, mean that the calculations carried out provide only an indicative estimate of the range of particle sizes produced.

2.7 Statistics

Data are presented as averages ± standard errors. One-way analysis of variance (ANOVA) was used to compare multiple groups of data; the *p* values from ANOVA are reported. Values were considered significantly different at the $p < 0.05$ level.

3. RESULTS

3.1 Root growth and Cd uptake

Results for hairy root growth with and without Cd are shown in Figure 1a. The control cultures without medium replacement exhibited a growth phase between Days 0 and 25 and produced approximately 0.58 g dry weight of roots at stationary phase. Addition of Cd relatively early in the growth period (5 days after inoculation) reduced the rate of root growth; however, the final biomass produced was similar to that in the control cultures. Cd addition after 14 days (mid-growth phase) or 25 days (late growth phase) had a negligible effect on growth. Consistent with the greater quantities of sugar and other nutrients available when the medium was replaced 14 days after inoculation, the growth period was extended in these cultures to 30–38 days and the total biomass produced was increased by about 38% to 0.80 g. Similar to the results obtained

without medium replacement, the addition of Cd at Day 14 at the time of medium replacement did not affect root growth.

In most of the cultures, initial high transient levels of Cd accumulation were observed in the root biomass in response to Cd addition (Fig. 1b). This was especially pronounced when Cd was added during the early stages of growth 5 days after inoculation. However, after about 25 days of culture, Cd concentrations in the roots were similar in all the cultures tested. Taking into account both the Cd concentrations and biomass levels produced, the total amounts of Cd accumulated in the roots of cultures treated with Cd without medium replacement were also similar irrespective of the time of Cd addition (Fig. 1c). However, when the roots were grown with medium replacement at Day 14, the total Cd accumulated by the biomass was up to 2-fold higher than for the other treatments.

The desired conditions for CdS nanoparticle synthesis are those that challenge the cultures so that Cd detoxification mechanisms are triggered, but are not harsh enough to damage the cells or impair growth. Dosing the cultures with Cd 5 days after inoculation was therefore eliminated as a production strategy because of the early detrimental effects on root growth. Because the total uptake of Cd was enhanced when the medium was replaced at the time of Cd addition, this protocol was used in subsequent culture experiments.

3.2 Extraction methods

Hairy roots treated with Cd 14 days after inoculation were harvested 4, 7 and 9 days later and subjected to four different extraction methods as described in Section 2.3. The amounts of Cd in the roots before extraction were 2.3 ± 0.16 µmoles, 2.2 ± 0.13 µmoles and 2.1 ± 0.001 µmoles, respectively for roots harvested 4, 7 and 9 days after Cd addition. The results for Cd recovery from the biomass are shown in Figure 2. The crude extracts in each case were brown in colour. For each of the extraction methods, there was no significant difference in yield with increasing treatment duration (p = 0.215–0.752). The highest Cd yields of 22–28% were achieved using a

combination of freeze-drying and freeze–thawing of the roots; these yields were a substantial improvement over the values of 8–15% found using the other three extraction methods (p = <0.001–0.002 depending on the duration of Cd treatment). Based on the results in Figures 1 and 2, in subsequent experiments, Cd was added to cultures with medium replacement 14 days after inoculation, the roots were harvested 9 days later, and the biomass was extracted using a combination of freeze-drying and freeze–thawing.

3.3 Properties of purified and size-fractionated extracts

The fractions collected after ion-exchange chromatography of the root extracts were analysed for their absorbance properties and Cd and inorganic sulphide contents (Figure 3). The optical properties of quantum crystals depends on the particle size; however, absorbance peaks at excitation wavelengths of 280–370 nm can be expected for 2–3 nm CdS nanocrystals [29–31]. As shown in Figure 3a, the absorbance peaks observed at 280 and 320 nm for roots treated with Cd were enhanced relative to those from control cultures without Cd treatment; the major absorbance peaks coincided roughly with a substantial increase in Cd content in fractions 35–50 eluted under KCl gradient conditions (Fig. 3b).

The UV–visible absorbance spectra of selected individual ion-exchange chromatography fractions are shown in Figure 4. UV transitions were evident for fractions 32–47 with absorbance peaks or shoulders in the 280–300 nm range. These optical properties are characteristic of small CdS nanoparticles. Selected ion-exchange chromatography fractions between 28 and 50 were pooled, freeze-dried and reconstituted with Milli-Q water to a volume of 2.5 mL for further purification and size-fractionation using gel filtration. As indicated in Figure 5a, substantial absorbance peaks and shoulders were observed for the gel filtration fractions after excitation at 280 and 320 nm. Absorbance Peak 1, which was observed with excitation at 280 nm but not at 320 nm, contained particles of relatively high molecular mass. Peak 1 corresponded to a peak in Cd content in fractions 25–50 but, on average, relatively low levels of inorganic sulphide (Fig.

5b). In contrast, absorbance Peak 2 (Fig. 5a) corresponding to gel filtration fractions 85–115 was observed at both excitation wavelengths tested and contained particles of smaller molecular mass. The spectral features of Peak 2 corresponded roughly to a Cd peak in fractions 100–115 that occurred together with substantial levels of inorganic sulphide (Fig. 5b).

The fluorescence properties of individual gel filtration fractions within Peaks 1 and 2 were examined at several excitation wavelengths between 300 and 340 nm to identify emission characteristics typical of CdS quantum dots. Typical fluorescence emission spectra for gel filtration fractions corresponding to Peak 1 in Figure 5a are shown in Figure 6: these spectra were obtained using an excitation wavelength of 310 nm. For fractions 28–47, the fluorescence intensity was negligibly low relative to root extracts from control cultures without Cd treatment. Although fraction 25 contained more highly fluorescent material than the other Peak 1 fractions, the fluorescence intensity was still low, suggesting that fluorescent CdS nanocrystals were not likely to be present in these samples. In contrast, gel filtration fractions corresponding to Peak 2 in Figure 5a exhibited high levels of fluorescence when irradiated at 300–340 nm; typical results at 310 nm are shown in Figure 7. Because larger particles are eluted before smaller particles during gel filtration, an increase in fraction number corresponds to a reduction in the size of the particles contained in the eluant. From the positions of the peaks in the spectra for the individual Peak 2 fractions, as the particle size decreased, the fluorescence peaks were blue-shifted to lower wavelengths or higher-energy emissions. This property is a characteristic of nanoparticles with quantum or semiconductor properties consistent with the presence of CdS quantum dots in gel filtration fractions 97–120. From the data in Figure 5b, the average ratio of inorganic sulphide to Cd in these fractions was 1.6 ± 0.17.

The results from estimation of CdS crystal size in gel filtration fractions 97–120 are shown in Table 1. As discussed in Section 2.6, these calculations provide an indicative estimate of the range of particle sizes produced; therefore, from the measured changes in fluorescence emission wavelength, the particle radii can be considered to fall broadly within the range of ~2–5

nm. We can say, therefore, that the fluorescence properties of the size-fractionated CdS nanoparticles produced by hairy roots are consistent with CdS quantum dots of diameter 4–10 nm. The particles fluoresced at wavelengths in the range 420–480 nm depending on particle size, corresponding to blue–violet on the colour spectrum.

Further experiments were conducted to test the photostability of the fluorescence emissions (Section 2.5.3) to confirm the effect of passivation of the nanoparticle surfaces. As shown in Figure 8 using gel filtration fraction 113 as a test sample, continuous excitation at 300 nm produced a small reduction in fluorescence intensity during the first 1 h of treatment, after which the emissions remained stable during the second 1 h of irradiation. Fluorescence emissions from control hairy root cultures without Cd are also shown; background fluorescence in the control cultures reflects the presence of photoactive compounds such as alkaloids and phenolics in the tomato hairy roots. These compounds are known to be very photostable [32] and, as the control emissions were constant during the first 1 h of irradiation, the control sample was not tested further.

3.4 Cd mass balance and nanoparticle yield

A mass balance was performed on Cd for the entire production and purification process to determine the overall yield of CdS nanoparticles in the gel filtration fractions and to monitor Cd selection and losses during the extraction and purification steps (Table 2). Of the 100 µM Cd or 26.6 µmoles Cd g^{-1} root dry weight added to the cultures, 17% was taken up by the biomass. The recovery of Cd after extraction using a combination of freeze-drying and freeze–thawing of the roots was about 19%. The two chromatography steps gave Cd recoveries of 69% and 81%; as phytochelatin complexes bind tightly to anion exchangers, the result for ion-exchange chromatography reflects the selection of Cd associated with phytochelatin peptides [33]. After gel filtration, the overall recovery of Cd was 5.0% of that initially taken up by the roots. As only those gel filtration fractions in Peak 2 of the chromatogram (Fig. 5a) exhibited optical/electronic

properties typical of CdS quantum dots, the yield of Cd in nanoparticles with semiconductor properties was only 1.4% of the total Cd in the biomass.

A mass balance on inorganic sulphide was also performed for the gel filtration stage of the purification process. As indicated in Table 2, a recovery of 103% was achieved, indicating that no losses of inorganic sulphide occurred during size-fractionation of the particles.

4. DISCUSSION

Cd- and sulphide-containing nanoparticles isolated from tomato hairy roots were found to exhibit the size and optical/electronic properties of CdS quantum dots. Synthesis of phytochelatin peptides capable of binding intracellular metal ions is a typical detoxification response of plants to heavy metal exposure. Incorporation of significant levels of inorganic sulphur into the metal–phytochelatin complex greatly enhances its metal-binding affinity; however, the extent to which this reaction occurs depends on the plant species and cultivar and other parameters such as the concentration of heavy metal [16]. Production of metal–sulphide–phytochelatin complexes that are restricted in size to the dimensions associated with quantum confinement effects can be expected to occur in plants even less frequently and may be rare. Demonstration in this work that such nanosized metallo-organic compounds are synthesised by tomato hairy roots provides a basis for future exploitation of this biomineralisation system for quantum dot production.

The major limitation encountered in this study for synthesis of CdS quantum dots was the relatively low yield of product achieved. Only about 1.4% of the Cd taken up by the hairy roots was associated with CdS nanoparticles that displayed appropriate size-related quantum dot properties (Table 2). This may be increased in the future by manipulating the root culture conditions; for example, raising the Cd and/or sulphide concentration in the medium, or optimising the times during the culture period for Cd addition and biomass harvest. Improvements are also required in the extraction procedures employed, as around 70–80% of the

Cd contained in the biomass was lost using the best extraction method tested (Fig. 2 and Table 2). Whereas, in previous work, freeze–thawing was used to selectively release CdS nanoparticles from yeast cells with low levels of contamination [34], the strong cellulosic walls of plant cells pose a more significant barrier to particle recovery. The proportion of extracted Cd occurring in Cd–phytochelain complexes was 69% (Table 2); this is towards the higher end of the range 2%–82% reported previously for different plant species [35]. Clustering or aggregation of quantum-sized CdS nanocrystals may also have reduced the final yield of semiconductor particles recovered from hairy root cultures. This is suggested by the presence of Peak 1 in Figure 5a, which was associated with particles of relatively high molecular mass containing elevated levels of Cd (Fig. 5b). The weak fluorescence emissions from these particles (Fig. 6) are consistent with the destruction of quantum effects with increasing crystal size due to aggregation. In contrast, particles of smaller molecular mass corresponding to Peak 2 in Figure 5a exhibited the optical/electronic properties of CdS nanocrystals of diameter 4–10 nm.

Photoexcitation of CdS semiconductor crystals under aerobic conditions leads to rapid photoinduced decomposition of the material accompanied by the release of sulphide ions [36,37]. The enhanced stability resulting from passivation of the crystal surface by coating with phytochelatin peptides was demonstrated for samples purified from hairy roots using irradiation tests with constant photoexcitation over 2 h (Fig. 8). Such stabilisation by native peptides to create an organo-metallic complex is a significant advantage associated with biological production of quantum dots; the passivation layer can also function as a biocompatible ligand for attachment of other organic molecules and markers. Although compounds similar to phytochelatins, such as glutathione and γ-glutamylcysteine, have also been shown to stabilise CdS nanocrystals to some extent, the beneficial effect is not as great as when full phytochelatin peptides are present as capping agents [37].

The average ratio of inorganic sulphide to Cd in the semiconductor CdS–phytochelatin complexes from hairy roots was 1.6 ± 0.17. This is similar to the ratio of 1.87 reported for Cd-

treated *Euglena gracilis* cells [38], but higher than the maximum values of 0.4–0.9 obtained previously in tomato plants, yeast and algae [14,16,39]. The relatively high level of sulphide incorporation in the hairy roots attests to the generation of an efficient and effective binding system for inactivation of intracellular Cd. The elevated ratio of inorganic sulphide to Cd in the nanoparticles may also reflect to some extent the production of sulphide complexes with other heavy metals normally present in plant cell culture media. Contamination of Cd–phytochelatin complexes with small amounts of Zn and Cu has been reported previously in hydroponically grown plants [40] and could also occur in *in vitro* plant culture systems. However, the concentrations of Zn and Cu in the Gamborg's B5 medium used in this work (7.0 and 0.10 µM, respectively) are small compared with the Cd dosing concentration of 100 µM. Moreover, as there is a metabolic requirement for Zn and Cu to support enzyme activity and other cellular functions, the intracellular availability of these metals would be restricted even further relative to Cd, so that their contribution to sulphide complexation in the roots is likely to be limited.

5. CONCLUSIONS

Tomato hairy root cultures treated with 100 µM Cd were used to produce CdS nanocrystals with quantum dot properties. Of four different biomass extraction methods tested, the highest Cd recovery was achieved using a combination of freeze-drying and freeze-thawing of the roots. Chromatographic selection of phytochelatin complexes and particle size-fractionation resulted in the isolation of 4–10 nm-diameter nanoparticles displaying the chemical and optical/electronic properties of CdS quantum dots. Fluorescence emissions from the particles were very stable during prolonged excitation, reflecting the passivating effect of the phytochelatin peptides. Improvements to the culture conditions and especially the biomass extraction methods are required to increase the yield of quantum dots produced using hairy roots.

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Table 1

Estimated sizes of CdS nanocrystals in gel filtration fractions.

Gel filtration fraction	Wavelength of fluorescence peak, λ (nm)	Energy of fluorescence emission, E (eV)	Band-gap energy shift, ΔE_g (eV)	Estimated particle radius (nm)
97	480	2.58	0.16	4.1
102	450	2.76	0.34	2.9
105	445	2.79	0.37	2.8
113	430	2.88	0.46	2.5
116–120	420	2.95	0.53	2.3

Table 2

Yields of Cd and inorganic sulphide at various stages during root culture and nanoparticle extraction and purification.

Stage	Cd introduced to the stage (μmoles g^{-1} root dry weight)	Cd recovered in the stage (μmoles g^{-1} root dry weight and %)	Cumulative Cd recovery relative to the amount of Cd taken up by the roots (%)
Uptake by hairy roots in culture*	26.6	4.51 17%	100%
Extraction**	4.51	0.84 19%	19%
Ion-exchange chromatography	0.84	0.58 69%	13%
Gel filtration (all fractions)	0.28	0.23 81%	5.0%
Gel filtration (Peak 2 fractions: Fig. 5a)	0.28	0.063 23%	1.4%
	Inorganic sulphide introduced to the stage (μmoles)	Inorganic sulphide recovered in the stage (μmoles and %)	
Gel filtration (all fractions)	0.29	0.30 103%	

* 100 μM Cd was added to hairy root cultures 14 days after inoculation with medium replacement at the time of Cd addition. The roots were harvested 9 days later.

** Using a combination of freeze-drying and freeze–thawing of the roots.

FIGURE CAPTIONS

Figure 1

Biomass growth and Cd uptake in hairy root cultures. **(a)** Growth of roots in cultures with: (●) no Cd added (control); (△) 100 µM Cd added 5 days after inoculation; (▼) 100 µM Cd added 14 days after inoculation; (○) 100 µM Cd added 25 days after inoculation; (■) no Cd added with medium replacement 14 days after inoculation; and (□) 100 µM Cd added 14 days after inoculation with medium replacement at the time of Cd addition. **(b)** Cd concentration in the biomass in cultures with: (△) 100 µM Cd added 5 days after inoculation; (▼) 100 µM Cd added 14 days after inoculation; (○) 100 µM Cd added 25 days after inoculation; and (□) 100 µM Cd added 14 days after inoculation with medium replacement at the time of Cd addition. **(c)** Total Cd (µmoles) in the biomass in cultures with: (△) 100 µM Cd added 5 days after inoculation; (▼) 100 µM Cd added 14 days after inoculation; (○) 100 µM Cd added 25 days after inoculation; and (□) 100 µM Cd added 14 days after inoculation with medium replacement at the time of Cd addition. The error bars represent standard errors from triplicate root cultures.

Figure 2

Cd recovery from hairy root biomass using four different extraction methods: chopping, freeze–thawing, a combination of chopping and freeze–thawing, and a combination of freeze-drying and freeze–thawing. The cultures were treated with 100 µM Cd 14 days after inoculation with medium replacement at the time of Cd addition; the roots were then harvested 4, 7 or 9 days later for Cd extraction. The error bars represent standard errors from triplicate root cultures.

Figure 3

Optical and chemical properties of 2-mL fractions collected after ion-exchange chromatography of hairy root extracts. **(a)** Absorbance measured at excitation wavelengths of 280 and 320 nm for

control cultures without Cd (dashed lines) and cultures treated with Cd (solid lines). A KCl gradient as shown was applied for elution of the fractions. **(b)** Amounts (μmoles) of (●) Cd and (○) inorganic sulphide in the fractions from cultures treated with Cd.

Figure 4

UV–visible absorbance spectra for individual fractions collected after ion-exchange chromatography of hairy root extracts. The absorbance was determined as the difference between the values observed for Cd-treated and control hairy root cultures. The labelled fraction numbers correspond to those in Figure 3. The transitions at 280–300 nm are characteristic of small CdS nanoparticles.

Figure 5

Optical and chemical properties of 1-mL fractions collected after gel filtration of selected and pooled ion-exchange chromatography fractions for cultures treated with Cd. **(a)** Absorbance measured at excitation wavelengths of 280 and 320 nm. The relationship between gel filtration fraction number and the average molecular mass (kDa) of particles in the fractions is indicated by the scale bar below the abscissa. **(b)** Amounts (μmoles) of (●) Cd and (○) inorganic sulphide in the fractions.

Figure 6

Fluorescence emission spectra for individual 1-mL gel filtration fractions from Peak 1 in Figure 5a. The fluorescence spectrum for fraction 25 is labelled; the other spectra with relatively low fluorescence intensity were generated by fractions 28, 31, 35, 39, 43 and 47. The excitation wavelength was 310 nm. The fluorescence intensity was determined as the difference in emission intensity between Cd-treated and control hairy root cultures. The relatively low levels of

fluorescence measured for the Peak 1 fractions suggest that CdS quantum dots were not present in these fractions.

Figure 7

Fluorescence emission spectra for individual 1-mL gel filtration fractions from Peak 2 in Figure 5a: **(a)** selected fractions in the range 113–120; and **(b)** selected fractions in the range 86–105. **(a)** and **(b)** have different ordinate scales. The excitation wavelength was 310 nm. The fluorescence intensity was determined as the difference between the emissions observed for Cd-treated and control hairy root cultures. For fractions 97–120, the emission peaks were blue-shifted to higher energies or lower wavelengths as the fraction number is increased, corresponding to a reduction in particle size. This relationship between particle size and emission wavelength is consistent with the presence of CdS quantum dots in these fractions.

Figure 8

Stability of fluorescence emissions from gel filtration fraction 113 irradiated at 300 nm and detected at 420 nm. The fluorescence intensity of the treated sample was reduced slightly during the first hour of irradiation but remained at a stable level during the second hour. Emissions from a control sample of hairy roots not treated with Cd are also shown.