Recombinant Fusion Protein PbrD Cross-Linked to Calcium Alginate Nanoparticles for Pb Remediation

Vidya Keshav,†§ Paul Franklyn,‡ and Kulsum Kondiah,‡*,†§

†School of Molecular and Cell Biology and ‡School of Chemistry, University of Witwatersrand, Private Bag 3, Wits, 2050 Johannesburg, South Africa
§Department of Biotechnology and Food Technology, University of Johannesburg, P.O. Box 17011, Doornfontein, 2028 Johannesburg, South Africa

ABSTRACT: Lead (Pb) pollution arising from industrial and mining activities has led to widespread environmental toxicity, particularly in South Africa. Humans exposed to Pb are reported to suffer from detrimental health impacts that can lead to fatalities. As such, there is an urgent need to remediate Pb from the environment. In this study, we propose the use of a Pb-specific recombinant fusion metalloprotein, rPbrD surface-cross-linked onto calcium alginate nanoparticles (CANPs) for the biosorption of Pb(II) from aqueous solution. The prepared biosorbents were characterized using scanning electron microscopy, transmission electron microscopy, and dynamic light scattering. Their ability to biosorb soluble Pb(II) was determined by inductively coupled plasma mass spectroscopy and their adsorption mechanism was described according to the Langmuir, Freundlich, Temkin, and Dubinin−Radushkevich adsorption isotherms. The rate of Pb uptake for bare CANPs and rPbrD-CANPs at a concentration of 100 mg/L metal was 3.34 and 8.82 mg/g, respectively, within 30 min. The adsorption data for the bare CANPs best fitted the Langmuir isotherm, whereas the adsorption data for rPbrD-CANPs best fitted the Freundlich isotherm. Based on the sorption intensity (n) and the separation factor (Rf), both biosorbents represent a favorable adsorption system. These findings suggest that the proposed nanobiosorbent is a promising candidate for the recovery of Pb ions present in high concentrations such as acid mine drainage or industrial effluent.

INTRODUCTION

Lead (Pb) toxicity is an environmental concern with a global impact on public health. Lead poisoning refers to the accumulation of the metal in the body, over time resulting in organ dysfunction, especially within the nervous system. It has caused permanent neurological damage in over 15 million children from developing countries. An increase in industrialization and urbanization has contributed to copious amounts of metals like Pb being discharged into natural water resources; rendering it unfit for human consumption. In South Africa, 40% of the population lives in rural settlements that depend entirely upon ground and surface water for domestic use. However, metal exposure is not only limited to communities in informal settlements but also the urban population when they consume the home-grown fruits and vegetables, which are sold as a source of income. Several reports highlight the presence of Pb(II) in South African river systems exceeding the acceptable limit of 10 μg/L. Fatoki and co-workers detected 240–1110 μg/L of Pb(II) in the Umtata River, Eastern Cape, while in the Western Cape, 30–40 μg/L of Pb(II) was recorded in Eerste River. Olujimi and co-workers reported an annual average of between 17.6 and 52.9 μg/L Pb(II) in the river systems of the Western Cape. Although new policies, regulations, and legislations have been made to control the decanting of industrial waste, ambiguities in the system still remain. Humphries and co-workers suggested that although a natural wetland in the Klip River, Gauteng acts as a sink for metals like Pb (source of contamination—Central Witwatersrand Basin), increase in contaminated discharge could compromise the wetland, itself becoming a source of metal contamination that would lead to devastating effects on the region’s water supply. Consequently, there is a focus on the development of effective strategies to remove or reduce the elevated concentrations of Pb(II) from wastewaters.

Remediation of Pb(II) from wastewaters using bacterial cells has been reviewed extensively as it offers a cheap alternative to chemical treatments. Although biosorption may offer high adsorption capacity and short reaction times, the system is inefficient in targeting specific metal ions when present in a complex medium. Peptides targeting specific metal ions (biopanning) have been attached to bacterial cells for Pb(II) remediation. However, introducing modified bacterial cells into water bodies is not yet feasible due to the uncertainty around their behavior in the environment.

In this study, we report the development of a novel nanobiosorbent that exploits the Pb(II) binding properties of the bacterial protein PbrD. PbrD is a metallochaperone found exclusively in Cupriavidus metallidurans CH34 that binds...
Pb(II) intracellularly, thereby reducing the cellular toxic effect of the metal at elevated concentrations. A recombinant form of PbrD (rPbrD) was immobilized onto calcium alginate (CA) nanoparticles (CANPs) and assessed for its ability to biosorb Pb(II) in vitro. Calcium alginate nanoparticles afford stability to the biomolecule in terms of temperature and pH, and enhance Pb(II) remediation, and are biodegradable. Herein, we report the use of a synthetic protein-based nanobiosorbent to adsorb Pb(II) at 25 °C. Furthermore, we assess the theoretical adsorption properties of the newly developed nanobiosorbent by the Langmuir, Freundlich, Temkin, and Dubinin–Radushkevich (D–R) isotherms owing to its application to remediate Pb(II) from wastewater.

**RESULTS AND DISCUSSION**

Characterization of rPbrD-CANPs. **Electron Microscopy.** Figure 1 shows the morphology and structure of bare CANPs and rPbrD-CANPs. Scanning electron microscopy (SEM) images for CANPs (Figure 1a) and rPbrD-CANPs (Figure 1b) reveal uniformly synthesized, monodispersed particles. Morphology of CANPs was of spherical nature, whereas rPbrD-CANPs were amorphous NPs. Transmission electron microscopy (TEM) micrographs further confirmed the formation of NPs with clear geometric boundaries. Bare CANPs with the size ranging from 5 to 30 nm were observed (Figure 1c) while rPbrD-CANPs appeared larger, between 60 and 85 nm in diameter (Figure 1d). The larger diameter of the rPbrD-CANPs was expected owing to the extended arm of glutaraldehyde and surface-cross-linked protein. When compared to the bare CANPs, rPbrD-CANPs remained in close proximity to each other. This can be attributed to the overall surface charge as well as low particle dispersion from reduced sonication that would otherwise lead to protein degradation.

**Protein Loading.** For the synthesis of the nanobiosorbent, two strategies used in the immobilization of rPbrD to the as-prepared CANPs were evaluated to determine which resulted in more efficient protein loading. When encapsulated within the CANPs, 66% of 5 μM rPbrD protein was loaded. However, when rPbrD was surface-cross-linked to the CANPs, 72% protein loading efficiency was achieved. The lower loading efficiency could be as a result of smaller pore size within the CANPs, which prevented the entry of rPbrD for binding. When compared to encapsulation, chemical cross-linking has been noted to not only improve thermal stability but also protein loading. All further characterization of the nanobiosorbent was performed with surface-cross-linked rPbrD-CANPs.
To improve the protein loading efficiency on surface-cross-linked CANPs, two additional parameters were assessed: the concentration of rPbrD and size of CANPs. Figure 2a shows that increasing the concentration of rPbrD from 5 to 40 μM did not increase loading efficiency. Similar trends were observed when the cellulase enzyme was immobilized onto calcium alginate beads,17 and bovine serum albumin (BSA) was encapsulated in chitosan nanoparticles.15 Both studies reported that an increase in protein concentration decreased the immobilization efficiency. The decrease in loading efficiency may occur due to the exclusion of binding to inner aldehydic groups by proteins diffusing from bulk solutions to the outer aldehydic chains, which are usually the first to be encountered.26 A high protein concentration leads to rapid immobilization resulting in partial or total blockage of the pore. Another contributing factor could be that at higher concentrations, recombinant proteins may precipitate out of solution becoming unavailable for immobilization.

In addition, changing the particle size (diameter) may affect the protein loading efficiency. Therefore, 5 μM rPbrD was loaded onto CANPs formed with different concentrations of CaCl₂. An increase in CaCl₂ concentration resulted in a corresponding increase in particle size. However, as the size of CANPs increased, a decrease in the protein loading efficiency was noted (Figure 2b). These findings suggested a reduction in the surface area of NPs and therefore reduced the number of protein binding sites. Similarly, a decrease in the CANP size (18 mM CaCl₂) slightly reduced the protein loading efficiency. In this study, CANPs synthesized with 22 mM CaCl₂ showed the highest loading efficiency of 74% and hence was characterized further and used for downstream applications. The stability of rPbrD cross-linked onto CANPs was monitored by measuring the protein concentration in the supernatant of the stored samples over a period of 7 days. There was no appearance of degeneration or leakage of the rPbrD protein in the supernatant, indicating the stability of the system.

Particle Size Distribution and ζ Potential. Particle size and surface charge of NPs are two important factors in their application due to their effect on dissolution. Dynamic light scattering (DLS) data indicated the particle size distribution of bare CANPs to be between 12 nm (±1.29) and 44 nm (±0.46) with an average polydispersity index (PDI) of 0.288 and rPbrD-CANPs to be between 50 nm (±2.57) and 140 nm (±1.95) with an average PDI of 0.326 (Figure 3a,c, n = 3). The peak of high intensity shows a relatively narrow size distribution typical of monodispersed NPs. The DLS data correlates with the data obtained from SEM images. The surface charge of NPs determines its ability to interact with other molecules. In this study, an average ζ potential of −26 mV (±0.85) and −31.1 (±0.3) were recorded for bare CANPs and rPbrD-CANPs, respectively (Figure 4a,b, n = 3).
The overall negative surface charge is due to the high content of carboxyl and hydroxyl groups of alginate. Immobilization of rPbrD to the surface of the CANPs results in increased particle stability. In addition, an increase in the $\zeta$ potential for rPbrD-CANPs was indicative of the success of protein immobilization by surface-cross-linking. These results suggest a stable colloidal system that would allow interaction with Pb(II) cations.

**Biosorption Efficiency of rPbrD-CANPs.** To determine the biosorption efficiency of the nanobiosorbent, both bare CANPs and rPbrD-CANPs were incubated separately in the presence of increasing concentrations of Pb(II) from 0.001 to 100 mg/L. The supernatant was analyzed using inductively coupled plasma mass spectrometry (ICP-MS) to indirectly detect the amount of bound Pb(II). Additionally, control samples containing the same concentration range of Pb(II) were prepared without the biosorbent and analyzed with ICP-MS. The control showed no change in the concentration of Pb(II). Figure 5 shows that the biosorption efficiency of rPbrD-CANPs increased (44–99%) with an increase in Pb(II) concentration (0.001–100 mg/L). At low concentrations of Pb(II), there is a surplus of active binding sites as the ratio of binding sites to metal concentration is high. These binding sites may include the carboxylate ($-\text{COO}^-$) and hydroxyl (OH) groups characteristic of CA particles as well as the cysteine residues of the protein rPbrD. A similar trend, where biosorption efficiency increased with an increase in the initial Pb concentration, was noted in other biosorption studies. This pattern may be attributed to an increased driving force for mass transfer, causing higher collisions between the sorbate and biosorbent, thereby leading to an enhanced uptake of Pb(II).

In addition to the apparent increase in the biosorption efficiency, the rate of Pb uptake for rPbrD-CANPs also significantly increased from $3.82 \times 10^{-5}$ to 8.82 mg/g with an increase in the Pb concentration (0.001–100 mg/L). This trend is attributed to the increased electrostatic attractions and mass transfer resistances between the Pb ions and the metal-binding sites. However, since this is the first study to report on a nanobiosorbent constructed from a recombinant protein, a true comparison with other reported nanobiosorbents is not possible.

Bare CANPs also showed an increase in Pb(II) uptake from 0.001 mg/L (74%) to 0.1 mg/L (97%) until saturation was obtained. Thereafter, the biosorption efficiency began to gradually decrease dropping to 37.85% at 100 mg/L Pb(II). The decrease in metal sorption at higher concentrations of Pb is attributed to the fully occupied metal-binding sites, where the biosorbent has reached a state of equilibrium. At this stage, there is constant sorption–desorption of metals ions, and therefore no further decrease in the residual metal concentration is observed unless the bound metals are desorbed by a change in solution parameters. This trend is typical of the adsorption Langmuir isotherm where the metals occupy the finite number of vacant adsorption sites in a monolayer formation, i.e., one metal ion per metal-binding site. These results are further confirmed with the adsorption isotherm data obtained for CANPs in this study. Similar results were obtained by Khezri and co-workers, who showed a 99% adsorption of bare CANPs in the presence of 25 mg/L Pb(NO$_3$)$_2$ and also discovered that as the initial metal ion concentration increased, the biosorption of Pb(II) decreased owing to the limited number of binding sites on CANPs. As expected, these results showed that both CANPs and rPbrD-CANPs are capable of binding Pb. However, when comparing the rate of uptake between the biosorbents, rPbrD-CANPs significantly biosorbed [Table 1, $p < 0.05$ analysis of variance (ANOVA)] more metal per gram of biomass compared to CANPs for all concentrations tested except at 0.001 mg/L. The increase in the rate of metal uptake in rPbrD-CANPs is due to the additional metal-binding sites available on rPbrD (cysteine residues) and its hypothesized specificity for binding Pb(II). At the highest concentration of Pb tested (100 mg/L), rPbrD-CANPs biosorbed almost all of the metals (99%) at a rate of 8.82 mg/g of nanobiosorbent, whereas CANPs biosorbed only 38% at a rate of 3.34 mg/g.

![Figure 4. $\zeta$ potentials of bare CANPs (a) and rPbrD-CANPs (b) indicating the formation of stable NPs.](image)

![Figure 5. Rate of metal uptake (mg/g) and corresponding biosorption efficiency (%) of bare CANPs and rPbrD-CANPs in the presence of increasing concentrations of Pb(II).](image)
nanobiosorbent. A significant difference in the rate of Pb uptake between CANPs and rPbrD-CANPs was observed for all concentrations tested, except at a concentration of 0.01 mg/L, where the rate of Pb uptake did not differ significantly (Table 1, \( p < 0.05 \) single-factor ANOVA). These findings suggest that while CANPs are able to remove Pb from aqueous solutions when present in low concentrations, adding rPbrD to the CANPs significantly enhances the biosorption efficiency even under high concentrations of the metal. This is ideal not only for South African natural water systems currently contaminated with Pb concentrations ranging from 17 to >1000 μg/L as mentioned earlier but also globally in countries like India, China, Mexico, and Brazil amongst others.33

The findings from the present study show that surface-cross-linked rPbrD-CANPs are good nanobiosorbents for Pb(II) that maintain high biosorption efficiency even in the presence of elevated concentrations of the metal. Consequently, the nanobiosorbent would be suitable to recover Pb ions from wastewater.

The binding of Pb(II) to rPbrD-CANPs was further confirmed by performing SEM with energy-dispersive X-ray spectroscopy (EDS) analysis on the washed nanobiosorbent pellet before (Figure 6a) and after incubation (Figure 6b) with the metal. The EDS spectra of rPbrD-CANPs post-biosorption showed a strong signal for Pb and weak signals for carbon (C), chloride (Cl), and oxygen (O) elements (Figure 6b). The peaks of Pb originated from the successful binding of Pb(II) onto rPbrD-CANPs while those of C, Cl, and O were attributed to the formulation of the CANPs itself.

**Adsorption Isotherms.** Adsorption isotherms were applied to understand the adsorption mechanism and compare the Pb(II) binding properties of rPbrD-CANPs to that of bare CANPs. For each isotherm, the values of the adsorption parameters and their respective linear regression line (\( R^2 \)) were calculated from the linear plots (Figure 7) prepared using the adsorption equilibrium data. The results of the adsorption isotherm values are presented in Table 2.

Table 1. Rate of Pb Uptake between CANPs and rPbrD-CANPs at Different Concentrations of Pb(II) (Single-Factor ANOVA)

| Pb conc (mg/L) | CANPs (\( Q_{\text{max}} \)) | rPbrD-CANPs (\( Q_{\max} \)) | sum of squared differences (SS) | degrees of freedom (df) | \( F \) | \( P \)-value | \( F \) crit |
|---------------|------------------|-----------------|-----------------|-----------------|----------------|---------------|---------------|
| 0.001         | 0.00006          | 0.00004         | 0.0010          | 1               | 76.90          | 0.00009*      | 7.71          |
| 0.01          | 0.00080          | 0.00083         | 0.0016          | 1               | 1.12           | 0.35          | 7.71          |
| 0.1           | 0.0085           | 0.0087          | 0.04            | 1               | 15.96          | 0.02*         | 7.71          |
| 1             | 0.08             | 0.09            | 12.90           | 1               | 17.58          | 0.014*        | 7.71          |
| 10            | 0.79             | 0.88            | 8772.53         | 1               | 898.37         | 0.0011*       | 18.51         |
| 100           | 3.34             | 8.82            | 29915 269.38    | 1               | 2692.68        | 0.0004*       | 18.51         |

*Significant difference \( p \leq 0.05.\)

Based on linear regression, bare CANPs best fit the Langmuir isotherm suggesting a monolayer formation of Pb(II). This indicates that the surface of CANPs contains a finite number of binding sites.19 The theoretical assumption correlates to the experimental data, where bare CANPs eventually reached saturation (at 0.1 mg/L Pb) and no further adsorption could occur. The linear regression data obtained for rPbrD-CANPs best fit the Freundlich isotherm, suggesting that Pb(II) binds in a multilayer formation, which is indicative of a nonrestrictive and exponential form of binding. In multilayer adsorption, the number of Pb(II) molecules is not necessarily identical to the exact number of active sites on the adsorbent surface due to the exponential adsorption of Pb ions, which occur due to interaction with other Pb molecules.34 As a result, the experimental data showed the complete removal of Pb(II) (100 mg/L) within 30 min when using a concentration of 11 g/L of nanobiosorbent (rPbrD-CANPs). Furthermore, adding...
rPbrD to the CANPs enhanced the maximum rate of adsorption of bare CANPs experimentally from 3.34 to 8.82 mg/g (rPbrD-CANPs). These findings are also supported by the maximum adsorption data (Q_max) obtained from the Dubinin–Radushkevich model with values for bare CANPs of 123.53 mg/g and rPbrD-CANPs of 598.60 mg/g of Pb(II)/g biosorbent, respectively. The differences between the adsorption capacity of bare CANPs and rPbrD-CANPs may be attributed to the different surface adsorption properties and particle size.20,27,28 The surface properties of rPbrD-CANPs differ from that of the CANPs due to the addition of rPbrD, which provides additional functional groups (amino acids) to the biosorbent. This changes the overall ionic strength and the pH of the biosorbent. Additionally, an increase in the particle size.20,27,28 The surface properties of rPbrD-CANPs are also attributed to the differences between the adsorption capacity values for all isotherms, no significant differences were observed. The adsorption process is exothermic, representing a physical adsorption system. Additionally, the calculated value of E (kJ/mol) obtained from the D–R isotherm was approximately 1 kJ/mol for bare CANPs and rPbrD-CANPs also, indicating a physisorption process.35 However, based on the low linear regression line for rPbrD-CANPs (R^2 = 0.3) obtained from the Langmuir and Temkin isotherm, no conclusive outcome can be made. Nevertheless, since rPbrD-CANPs indicated a multilayer coverage, and based on the E value from the D–R isotherm, the interaction between Pb(II) and the proposed sorbent is most likely through a physisorption process.

## CONCLUSIONS

In this paper, we report the synthesis and characterization of a novel recombinant protein-based lead (Pb) biosorbent for potential application in wastewater treatment. The metallochaperone PbrD was overexpressed and surface-cross-linked to CANPs to form spherical, monodispersed particles with an average diameter of 80 nm. The negative surface charge of the nanobiosorbent indicated a stable colloidal system with the ability to bind positively charged Pb(II). Although bare CANPs were able to bind Pb(II), the rPbrD-CANP nanobiosorbent showed enhanced uptake of the metal (99.9%) at a rate of 8.82 mg/g biosorbent even in the presence of elevated concentrations. The adsorption by rPbrD-CANPs best fitted the Freundlich and Dubinin–Radushkevich isotherms indicating that Pb(II) binding occurred by physical sorption in a multilayer formation. Further development of the nanobiosorbent will include optimizing its performance for real wastewater treatment systems could provide a suitable treatment strategy for the recovery of Pb(II) present from 1 μg/L to 100 mg/L, which covers the variability of metal concentration in wastewater effluent.

## MATERIALS AND METHODS

**Materials.** Sodium alginate (SA, low viscosity grade; 15–25 cP, 1% H_2O), calcium chloride (CaCl_2), glutaraldehyde (70%), and lead nitrate (Pb(NO_3)_2) were purchased from Sigma-

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**Table 2. Adsorption Parameters of the Langmuir, Freundlich, Temkin, and Dubinin–Radushkevich Isotherms for the Adsorption of Pb(II) onto Bare CANPs and rPbrD-CANPs**

| adsorption parameters | CANPs     | rPbrD-CANPs |
|-----------------------|-----------|-------------|
|                       | Experimental Adsorption Capacity |           |
| Q_{max} (mg/g)        | 3.34      | 8.82        |
| Langmuir Isotherm     |           |             |
| Q_{max} (mg/g)        | 0.24      | 0.13        |
| K_L (L/mg)            | 5.49      | 7.16        |
| r^2                   | 0.9539    | 0.378       |
| Freundlich Isotherm   |           |             |
| 1/n                   | 0.84      | 2.06        |
| n                     | 1.19      | 0.49        |
| K_F (mg/g)            | 1.03      | 1.01        |
| r^2                   | 0.9134    | 0.8772      |
| Temkin Isotherm       |           |             |
| A_t (L/mg)            | 2.18      | 1.15        |
| B_t                   | 9.98      | 2.55        |
| B (J/mol)             | 248.3     | 972.7       |
| r^2                   | 0.7735    | 0.324       |
| Dubinin–Radushkevich Model |           |             |
| Q_0 (mg/g)            | 123.53    | 598.60      |
| K_d (mol/L^2)         | 6 x 10^-7 | 1 x 10^-6   |
| E (J/mol)             | 912.87    | 707.106     |
| r^2                   | 0.6412    | 0.8108      |

**Table 3. Separation Factor of the Biosorbents Obtained from the Langmuir Isotherm**

| initial [Pb(II)]  | R_L (CANPs) | R_L (rPbrD-CANPs) |
|-------------------|-------------|-------------------|
| 0.001             | 0.99        | 0.99              |
| 0.01              | 0.95        | 0.93              |
| 0.1               | 0.65        | 0.58              |
| 1                 | 0.15        | 0.12              |
| 10                | 0.02        | 0.01              |
| 100               | 1.80 x 10^-3| 1.4 x 10^-3       |
Aldrich (Missouri) and used without further purification. The experimental procedures were performed at ambient temperature (≈25 °C) unless otherwise stated. All solutions were prepared with distilled water obtained from the Elix Essential Water Purification System (Merck, Germany).

**Preparation of Recombinant PbrD Protein (rPbrD).** The PbrD protein was expressed in *Escherichia coli* BL21 (DE3) cells carrying the constructed vector pPET32Xa/LIC-pbrD. This vector was constructed to carry the full-length pbrD gene fused to an N-terminal Trx-Tag, His-Tag, and STag; the resulting fusion protein is hereafter referred to as rPbrD. Optimal expression of rPbrD (∼50 kDa) was achieved at 37 °C, 5 h post-induction with 1 mM isopropyl β-D-1-thiogalactopyranoside. Overexpressed rPbrD was purified by Ni-NTA affinity chromatography under denaturing conditions using 8 M urea. Purified protein (>85%) was eluted in elution buffer (50 mM NaH2PO4, 300 mM NaCl, 8 M urea, 400 mM imidazole, pH 8) and refolded by dialysis into buffer containing reduced concentrations of urea and dithiothreitol (DTT) (50 mM Tris, 0.5 M L-arginine, 1 M urea, 10% (v/v) glycerol, 1 mM DTT, pH 8). The refolded protein was further purified by dialysis into phosphate-buffered saline buffer [containing 0.5 M L-arginine, 1 mM DTT, 10% glycerol (v/v), pH 8] and used for immobilization onto CANPs.

**Preparation of CANPs.** Calcium alginate nanoparticles were synthesized according to the method described by Daemi and Barikani35 with slight modification. A solution of 0.06% (w/v) SA was prepared in distilled water under constant agitation and heated to 60 °C until the polymer was completely dissolved. Simultaneously, 22 mM CaCl2 solution was prepared by dissolving in distilled water and titrated into the SA solution at a flow rate of 0.05 mL/min using a peristaltic pump (EP-1 Econo Pump, Bio-Rad) under continuous homogenization. The solution was further agitated for 1 h to achieve the complete formation of the NPs. The homogenized solution was centrifuged at 15 000 g for 10 min (Heraeus Multifuge X1R Centrifuge, Thermo-Fisher Scientific) to remove impurities. The pellet was reconstituted in 10 mL distilled water and pulse-sonicated with three cycles of 15 s each at 50% amplitude (Q125 sonicator, Qsonica) for even dispersion of prepared NPs.

**Preparation of rPbrD-CANPs.** Purified rPbrD was either encapsulated within or surface-cross-linked to the CANPs using glutaraldehyde as a cross-linker.

**Preparation of Encapsulated rPbrD-CANPs.** Separate solutions of 0.06% (w/v) SA and 22 mM CaCl2 were prepared as described earlier. Glutaraldehyde solution (0.5%) was added to the dissolved SA solution and agitated for 30 min at room temperature. Thereafter, 5 μM rPbrD protein was added to the solution and further agitated for 30 min to allow maximum binding of the protein. To form nanoparticles, prepared CaCl2 solution was titrated into the polymer solution containing rPbrD as described earlier and was further agitated for 1 h. The homogenized solution was centrifuged at 15 000g for 10 min to remove impurities, and the supernatant containing unbound protein was quantified to determine protein loading efficiency using the Qubit protein assay kit together with the Qubit Quantification Platform fluorometer (Thermo Scientific) as previously described. The amount of protein loaded on surface-cross-linked CANPs was found to be consistently higher in replicate preparations than for encapsulated CANPs. Subsequently, further development of the nanobiosorbent was based on the surface-cross-linked method to prepare rPbrD-CANPs.

**Protein Loading Efficiency of rPbrD-CANPs.** To obtain maximal protein loading efficiency of rPbrD onto the CANPs, protein concentration and nanoparticle size were varied. Several concentrations of the protein (5, 10, 20, and 40 μM) were surface-cross-linked to the as-prepared CANPs and incubated for 30 min. The solution was centrifuged at 15 000g for 10 min, and the concentration of the unbound protein in the supernatant was quantified using the Qubit Quantification Platform fluorometer (Thermo Scientific) as previously described. Alternatively, CANPs of variable sizes were prepared using different concentrations of CaCl2 (18, 26, 30, and 36 mM) with a standard concentration of 5 μM rPbrD and evaluated for protein loading. All samples were prepared in a minimum of triplicates, and the results reported are representative of the mean ± SD.

**Characterization of CANPs and rPbrD-CANPs.** Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDS). Bare and rPbrD-CANP samples (10 μL) were mounted onto a carbon-coated aluminum stub and placed in a desiccator to dry for 24 h. The prepared stubs were then sputter-coated twice with carbon and once with gold palladium and analyzed using the FEI Nova NanoLab 600 FEG-SEM/FIB (FEI) operated at 30 kV coupled to the EDS. Samples were prepared in triplicates, and images were recorded in a minimum of triplicates.

**Transmission Electron Microscopy (TEM).** Twenty microliters of bare CANPs or rPbrD-CANPs were placed onto a copper mesh grid and dried at room temperature for 24 h. TEM measurements were performed on an FEI Tecnai T12 instrument (FEI) operating at 120 kV.

**Particle Size Distribution and ζ Potential.** Dynamic light scattering (DLS) for particle size distribution and ζ potential measurements was carried out on a Zen 3600 Laser Particle Size Analyzer (Malvern Instruments). Samples were pulse-sonicated at a low amplitude of 15%, and 1 mL sample was transferred into a sterile cuvette for analysis at a wavelength of 532 nm. All measurements were performed at 25 °C. For each preparation, an average of three separate measurements was reported, and the results are representative of the mean ± SD.

**Pb Binding Assay with CANPs and rPbrD-CANPs.** Biosorption studies were conducted using 11 g/L of CANPs and rPbrD-CANPs, respectively. The biosorbents were incubated separately with increasing concentrations of Pb(NO3)2 solution (pH 8) ranging from 0.001 to 100 mg/L for 30 min under constant agitation (150 rpm) to facilitate Pb(II) binding. The samples were then centrifuged at 24 000g for 20 min. The supernatant containing unbound Pb(II) was collected, adjusted with 1% nitric acid, and analyzed using an Agilent 7900 inductively coupled plasma mass spectrometer (ICP-MS, 208 Isotope) (Agilent Technologies, Inc.) at the Council for Scientific and Industrial Research (South Africa).
to indirectly detect the concentration of bound Pb(II). Appropriate controls using Pb(NO₃)₂ solutions of similar concentrations (0.001–100 mg/L) without the addition of nanoparticles were included. The adsorption experiments were performed in triplicate, and an average of three measurements are reported. The percentage of Pb removal (biosorption efficiency) and the adsorption capacity (Qₑ) (mg/g) were determined using eqs 1 and 2, respectively:

\[
\text{biosorption efficiency} (\%) = \left( \frac{C_i - C_e}{C_i} \right) \times 100
\]

\[
\text{adsorption capacity (mg/g)} = \frac{(C_i - C_e)V}{w}
\]

where \(C_i\) is the initial metal concentration (mg/L), \(C_e\) is the residual metal concentration (mg/L), \(V\) is the solution volume (L), and \(w\) is the amount of adsorbent in solution (g).

**Statistical Analysis.** Statistical hypothesis testing was performed on the biosorption data (rate of metal uptake) using the statistical program R, version 3.4.3. The single-factor ANOVA analysis was used to compare the rate of Pb uptake between CANPs and rPbrD-CANPs for each concentration evaluated in this study. All experiments were performed in triplicate to ensure statistical accuracy. A statistical difference was considered significant when the p-value (α-level) is ≤ 0.05 (95% confidence).

**Adsorption Isotherm.** Modeling experimental adsorption data of new biosorbents is essential to provide a proper understanding and interpretation of the adsorption mechanisms they follow. Consequently, their commercial potential and application feasibility can be evaluated. Therefore, the four frequently reported adsorption isotherms (Langmuir, Freundlich, Temkin, and Dubinin–Radushkevich) were used to model the adsorption mechanism of both bare and rPbrD-CANPs in the presence of Pb(II) for the purpose of this study. However, it is crucial to apprise that the data obtained from these isotherms are only theoretical and not experimental and therefore are purely descriptive and used as a guide for downstream application.

The following summarizes the different adsorption isotherms, linear equations, and adsorption properties, which were applied using the adsorption equilibrium data from the biosorption study.

**Langmuir Isotherm.** The Langmuir adsorption isotherm was initially developed to study the gas–solid-phase adsorption onto activated carbon. This isotherm depicts a homogeneous sorbent surface containing a limited number of metal-binding sites that are similar in size and shape. If the experimental data best fit the Langmuir isotherm, then the following is predicted: Pb(II) forms a monolayer coverage on the surface of CANPs or rPbrD-CANPs and no further adsorption occurs. The isotherm further predicts the adsorption intensity related to the bonding energy and \(n\) is the adsorption intensity between Pb and sorbent.

**Temkin Isotherm.** The Temkin adsorption model considers the effects of indirect adsorbate–adsorbate interactions during the adsorption process and best fits with a monolayer coverage. Considering only the intermediate ion concentrations, the model assumes that as metal ions bind and cover the surface of the biosorbent, the heat of adsorption would decline linearly rather than logarithmically. If the experimental data best fit the Temkin isotherm, then the following is predicted: a positive B (heat) value would indicate that Pb(II) binds to CANPs or rPbrD-CANPs in an exothermic process, whereas a negative value would indicate an endothermic process. The adsorption parameters \(A_t\) and \(B_t\) were determined from the slope and intercept of the \(Q_e\) versus \(\ln C_e\) plot based on the linearized equation presented as eq 5:

\[
\log Q_e = \log K_f + \frac{1}{n} \log C_e
\]

where \(K_f\) is the Freundlich adsorption constant (mg/g) relating to the bonding energy and \(n\) is the adsorption intensity between Pb and sorbent.

**Dubinin–Radushkevich isotherm.** The Dubinin–Radushkevich (D–R) adsorption isotherm is an empirical isotherm which indicates the sorption energy \(B_{DpR}\) distribution between metal ions and the biosorbent. The physisorption prediction best fits a multilayer coverage displaying van der Waals forces of interactions, whereas the chemisorption process best fits monolayer coverage displaying covalent
bond formation. If the experimental data best fit the D–R isotherm, then the following is predicted: If a low enthalpy (<20 kJ) is measured, then the nature of bonding between Pb(II) and CANPs or rPbrD-CANPs is a physisorption process. Similarly, a high enthalpy (>200 kJ) would depict a chemisorption process. The adsorption parameters (Qe and Ks) were determined from the slope and intercept of the ln Qe versus ε2 plot based on the linearized equation presented in eqs 8–10:

\[
\ln Q_e = \ln Q_{e0} - K_{\text{ad}} \epsilon^2 \\
\epsilon = RT \ln \left(1 + \frac{1}{C_s}\right) \\
E = \frac{1}{\sqrt{2 B_{DR}}} 
\]

where \(\epsilon\) is the Polanyi potential, Qe is the theoretical saturation capacity (mg/g), Ks is the D–R adsorption energy constant (mol\(^{-2}\)kJ\(^{-1}\)), and BDR is the isotherm constant related to the free mean sorption energy.

**AUTHOR INFORMATION**

**Corresponding Author**

*E-mail: kuslumk@uj.ac.za. Tel: +27 011 559 6102.

**ORCID**

Vidya Keshav: 0000-0002-7785-5280

Kulsum Kondiah: 0000-0003-4174-7496

**Notes**

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