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PAPER

Genetically engineered polypeptides as a new tool for inorganic nano-particles separation in water based media

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The present paper relates a method for the separation of an insoluble inorganic powder out of a mixture of several insoluble powders with different chemical compositions, using genetically engineered inorganic binding peptides (GEPI). GEPI are small peptides that recognize and specifically bind an inorganic solid material. This GEPI is anchored to magnetic beads for easy recovery of the powder of interest from the mixture.

1. Introduction

Specificity is a hallmark of biological interactions. In natural systems, biomolecules are able to differentiate individual target molecules from thousands of competitors. Mimicking this specificity represents a challenge in inorganic systems where the target is diffuse and inseparable from a large competing background.[1–3] Genetically engineered peptides for inorganics are small peptides that specifically and selectively bind an inorganic surface. Their high specificity depends on (i) the chemical composition, (ii) the structure (powder or flat surface) and the (iii) crystallographic form of the inorganic target substrate.[4] Inorganic binding peptides have, for example, been shown to be able to recognize one crystallographic form in a mixture of Au polycrystalline powder.[5] This high specificity gives them interesting properties and several possible applications have already been highlighted in bio-nanotechnology, medicine or microelectronics.[3,6–11] Inorganic-binding peptides may also be used for surface quality control measurements[12] and crystal defects recognition.[13]

Although research has been directed toward a general understanding of protein binding to a solid, it is not yet clear how peptides recognize an inorganic surface. In general, the specificity of a peptide for a surface may originate from both chemical (hydrogen binding, polarity and charge effect) and physical (conformation, size and morphology) recognition mechanisms. For a given peptide–inorganic substrate combination, all these parameters may contribute to the interaction mechanism to varying degrees depending on the peptide sequence, chemistry and topography of the solid surface and the conditions of the solvent.[2,14,15] The molecular architecture (conformation) of the peptide at the surface of its inorganic target governs the specificity of the interaction since amino acid sequence itself, not the content of amino acid, plays the major role in peptide molecular recognition of solid surfaces. In a recent paper, Heinz *et al.* gave more insight about the importance of peptide conformation on the surface using molecular dynamic and valence force field.[16] Computing energies, free energies and entropies of adsorption of solute molecules on a given surface, they showed that strongly binding peptides often assume a flat 2D conformation so that most residues are in direct contact with the metal surface and weakly binding peptides retain more features of the preferred 3D conformation in solution.

The present paper highlights a still unexplored application for inorganic binding peptides, a process for sorting insoluble inorganic individual components out a blend of inorganic powders on the basis of their chemical composition, morphology, or crystallographic form. Moreover, this method is suitable for the separation of any type of solid material providing that a GEPI recognizing the material and form of interest is available. The blend to be sorted may comprise inorganics only or a mixture of both inorganic powder particles and organic materials provided the entity to be sorted is not water soluble. The results we report were obtained with powder particles of sizes <5 μm.

Current methods for inorganic material particles sorting like filtration, electrically assisted cross-filtration, sedimentation, centrifugation or flotation are affected by several technical constraints limiting their fields of application. Indeed, filtration processes (at room or high temperature) require a difference in particle size or/and morphology[17] and is not selective of material chemistry. To overcome this limitation, electrically assisted cross-flow filtration, based on particle surface charge, has been developed and it allows separation of inorganic powders in function of the chemical nature of the material.[18] However, the efficiency of this process depends on a lot of parameters such as pH, ionic strength, conductivity, particle size or nature of the particles. Industrial implementation of all pre-cited processes is time consuming. Sedimentation and centrifugation processes are

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based on the formation of several layers with different particle size and density.[19,20] For example, in the case of silicium recovery, 90.8% purity Si is obtained with a maximum yield of 74.1%. However, pure powders of particle size inferior to 10 μm cannot be obtained.[21] A flotation process for separating small mineral particles has been studied for more than 60 years.[22,23] However, this technique greatly depends on the size and surface charge of the particles and is not easily applied to powders with particle size lower than 10 μm.[24]

The present method is simple, fast and performed in aqueous media at room temperature. Once the GEPI recognizing the solid material of interest has been obtained, the separation method itself comprises three steps: (1) the dispersion of the powder mixture, (2) contacting the powder mixture with the inorganic-binding peptide specifically binding to the inorganic constituents of interest and (3) recovering the GEPI bound powder particles. This present method has yielded a patent (R1943 PCT) entitled 'Inorganic-binding Peptides and Quality Control Methods using them'.

For the present sorting experiment of a binary blend of insoluble metallic oxides, an inorganic-binding peptide has been covalently grafted to magnetic beads before being contacted with the powder articles. After exposure of GEPI-magnetic beads to the blend of inorganic particles, the beads can be retrieved by magnetisation, dragging down individual inorganic constituents with them. In order to complete the separation of the powder particles from the mixture, the binding interaction between the inorganic-binding peptide and its substrate is weakened *via* acidic treatment, releasing the inorganic particles.

This "biologically assisted" sorting process can lead to a technological breakthrough in the field of insoluble solid powders and particles separation, resulting in a wide array of applications in numerous sectors such as water treatments (small inorganic particles can absorb dangerous compounds[25,26]), recycling of solar silicon wastes (to obtain high silicon grade from wastes[20]) and in the mining industry for the separation of mineral phases containing specific ions such as lithium, which can be used for Li-ion batteries.[27]

2. Experimental

2.1 Materials

ZnO (99.99%) and Cu_2O powders were purchased from Sigma-Aldrich (Ref 255750 and 12841). The ZnO-binding peptide VRTRDDARTHRK identified by Kjaergaard *et al.* in 2000 (ref. 28) was synthesized by Genescript (Piscataway, USA) with a fluorescein dye attached to its *N*-terminal end and with its *C*-terminal end amidated. Dynabeads M-270 Epoxy magnetic beads were purchased from Invitrogen (Merelbecke, Belgium).

2.2 Media

Tris, NaCl, BSA and Tween® 20 were purchased from Sigma-Aldrich (Germany). A 50 mM Tris buffer, 1.5% Tween® 20, and 15 mM NaCl (pH = 7.6) was used as the binding buffer for all experiments. A 0.1 M Tris HCl buffer (pH 2.2) was used as the elution buffer.

2.3 Inorganic-binding peptides preparation

Peptides were solubilized in 50 mM Tris, 1.5% Tween® 20, 15 mM NaCl (pH = 7.6) binding buffer at a concentration of 0.1 mg mL^{-1}.

2.4 Inorganic powders preparation

10 mg of ZnO and Cu_2O powders were washed 3 times with 1 mL of binding buffer. After each wash, powders were recovered by centrifugation and re-suspended in fresh binding buffer volume. After washing, powder suspensions were sonicated at 30% for 5 minutes (ultrasounds source integrated in the Malvern Mastersizer 2000).

2.5 Powder characterization

Particle size distribution (Malvern Mastersizer 2000) was measured on powders (10 mg mL^{-1}) dispersed in 50 mM Tris, 1.5% Tween 20, and 15 mM NaCl (pH = 7.6) binding buffer before or after sonication (power 30%, duration 5 min). Particle size distribution was also measured on the same powders one hour after sonication.

2.6 Dynabeads M-270 magnetic beads functionalization with ZnO-binding peptide

Dynabeads M-270 magnetic beads are composed of highly cross-linked polystyrene with iron oxide magnetic material precipitated in pores evenly distributed throughout the particles. The beads are further coated with a hydrophilic layer of glycidyl ether (epoxy) functional groups. Dynabeads M-270 Epoxy (6 mg, 4×10^8 beads) were functionalized with the fluorescein-labelled ZnO-binding peptide (120 μg) according to the manufacturer procedure (Invitrogen). The amino group of the lysine of the ZnO-binding peptide reacts with the epoxy group on the surface of the magnetic beads. Unbound peptides are discarded by several washing and magnetisation steps.

2.7 Binding studies between the inorganic binding peptides and the inorganic powder

After washing, ZnO and Cu_2O powders were saturated for 2 h with 1% BSA in binding buffer. After saturation, the ZnO-binding peptide (0.1 mg mL^{-1}) was mixed in binding buffer for 1 h with each inorganic powder separately, washed overnight with the same buffer, and finally observed by fluorescence microscopy in order to check that the peptide specifically recognizes the ZnO powder.

2.8 Sorting of a binary blend of insoluble metallic oxides (Fig. 4)

ZnO-binding peptides functionalized beads were mixed for 1 h at room temperature with washed and sonicated ZnO and Cu_2O powder blends in the respective amounts described in Table 1. After incubation, beads are recovered by magnetisation and washed with binding buffer (Fig. 4C). This washing/separation step was repeated 10 times and washing fractions are pooled. Inorganic particles bound to peptide functionalized beads were eluted with a Tris HCl elution buffer and the beads separated by

Table 1 Atomic absorption analyses of Cu and Zn amount (mg L^{-1}) in the initial mixture, the pool of washed fractions and the pool of eluted fractions after ZnO insoluble powder separation from a ZnO–Cu_2O mixture *via* ZnO inorganic binding peptides bound to magnetic beads

Experiment 1	Zn/mg L^{-1}	Cu/mg L^{-1}	Mass ratio Zn/Cu	% Zn	% Cu	% Ratio Zn/Cu
Initial amount	900	4415	0.2	100	100	1
Pool of washed fractions (10)	421	5319	0.08	46.7	120.4	0.38
Pool of eluted fractions (2)	543	154.1	3.52	60.2	3.4	18
Experiment 2	Zn/mg L^{-1}	Cu/mg L^{-1}	Mass ratio Zn/Cu	% Zn	% Cu	% Ratio Zn/Cu
Initial amount	3450	5400	0.64	100	100	1
Pool of washed fractions (10)	1336	5572	0.24	40.1	103	0.39
Pool of eluted fractions (2)	1825	49.5	36.9	52.8	0.9	59

magnetisation. The elution step was repeated twice and elution fractions pooled.

2.9 Atomic absorption spectrometry

Zn and Cu were quantified in initial, washing and elution fractions by Atomic Absorption Spectrometry on a novAA300 Spectrometer (Analytik Jena). Samples were first solubilized in 20% HCl and diluted 2000 times in several steps with a Hamilton dilutor machine. Calibration was done with three ISO standards. Zn and Cu amounts were reported in mg L^{-1} and converted to percentage.

2.10 Microscope analyses

Before and after binding to ZnO-binding peptide labelled with fluorescein, powders were examined by optical microscopy with fluorescence. Confocal imaging was performed using a Leica TCS SP2 inverted confocal laser microscope (Leica Microsystems, Germany). Digitized images were acquired using a 63× (NA 1.2). Fluorescein was visualized by using an excitation wavelength of 488 nm and the emission light was recorded at 535 nm. The acquisition was set up to avoid any cross-talk of the three fluorescence emissions. A series of optical sections were carried out to analyze the spatial distribution of fluorescence, they were recorded with a *Z*-step ranging between 1 and 2 μm.

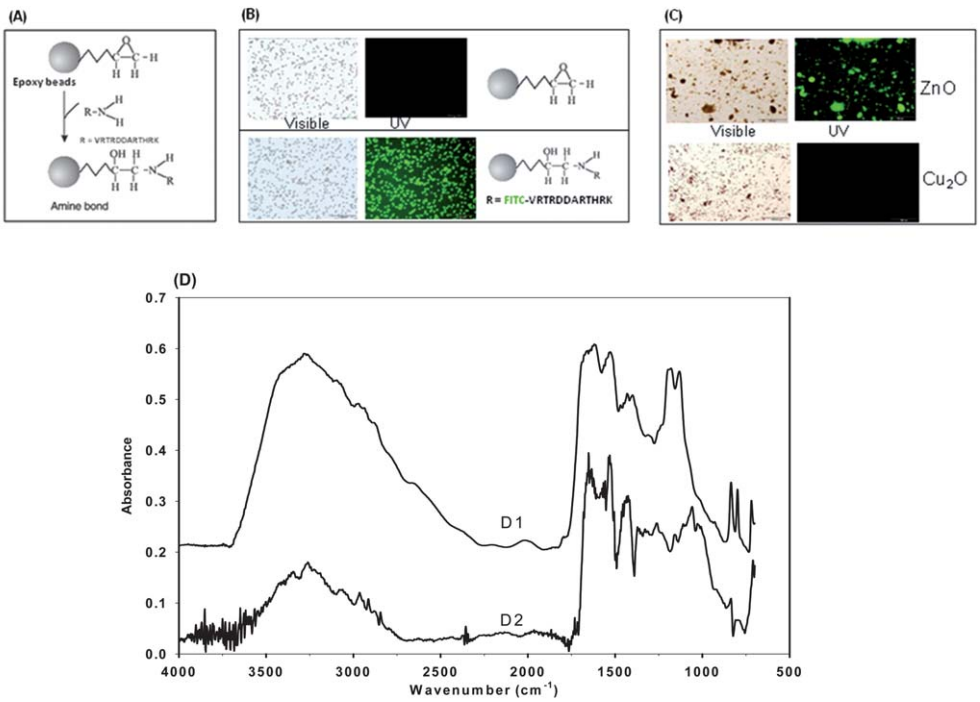


Fig. 1 (A) Schematic representation of epoxy magnetic beads functionalization with ZnO-binding peptide. A covalent link is formed between the epoxy group of the beads and the *N*-terminal end of the ZnO-binding peptide. (B) Microscopic analyses under visible and UV light of epoxy magnetic beads (up) and epoxy magnetic beads grafted with GEPI ZnO–FITC. (C) Microscopic analyses of the GEPI ZnO-FITC peptide in interaction with ZnO (up) and Cu_2O (down) powders dispersed in Tris 50 mM, NaCl 15 mM, Tween 20 1.5%, pH 7.6. Magnification VIS (40×), UV (40×, 2 s). (D) Micro-FTIR of (D1) the pure peptide spectrum (shifted of 0.2 absorbance units for a better view) and (D2) the difference of absorbance IR spectra between the ZnO powder *with ZnO-binding peptide and without peptide*, in the binding buffer and further washed with SDS and overnight with water.

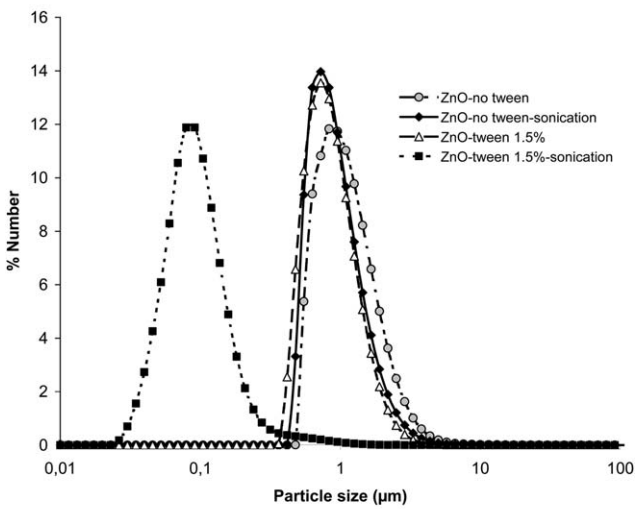
Image processing, including background subtraction, was performed with Analys software (version 2.5).

ZnO and Cu_2O powders were also observed by scanning electron microscopy (ESEM Philips XL 30) under 8000× magnification.

2.11 FTIR analyses

To further prove that the peptide is indeed bound to the ZnO, transmission FTIR spectra were recorded on small particles (60 μm) deposited on a monocrystalline silicium plate with the help of a microscope (IRScope II) coupled to an Equinox 55 Bruker FTIR spectrometer. Measurements were made on the ZnO powder incubated for 2 h with ZnO-binding peptides in the binding buffer. A washing protocol has been set up in order to keep peptides specifically bound to the ZnO particles and to remove the Tris and Tween 20 components of the binding buffer that may mask the peptide signals in the IR spectra. To that aim, after peptide binding, the ZnO powder has been washed with SDS 0.01% for 2 h (SDS tends to form mixed micelles with the Tween) and then overnight with water. Two controls have also been analyzed by IR: (i) the ZnO powder incubated with the binding buffer (no ZnO-binding peptide) and washed according to the protocol described above and (ii) the ZnO-binding peptide alone.

(A)



(B)

Sample names	Particle size (μm)		
	d(0.1)	d(0.5)	d(0.9)
ZnO-no tween	0.59	0.67	1.92
ZnO-no tween-sonication	0.53	0.8	1.53
ZnO-1.5 wt.% tween	0.49	0.76	1.38
ZnO-1.5 wt.% tween-sonication	0.05	0.08	0.16
Cu_2O-1.5wt.% tween-sonication	1.06	1.58	2.76

Fig. 2 (A) Particle size distribution (% N) of ZnO powder (10 mg mL^{-1}) in Tris 50 mM buffer, pH 7.6 (grey circles), in Tris 50 mM buffer, pH = 7.6 + sonication (30%, 5 min) (black lozenges), in Tris 50 mM, Tween 20 1.5%, pH = 7.6 (white triangles), in Tris 50 mM, Tween 20 1.5%, pH = 7.6 + sonication (30%, 5 min) (black squares). The dispersion is stable and particle size distribution curves remain unchanged 1 h after sonication. (B) ZnO and Cu_2O particle size (μm) in Tris buffers with or without Tween and sonication.

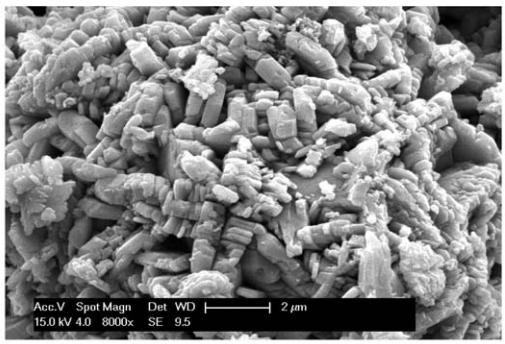
3. Results and discussion

In the present paper, inorganic binding peptides were used in an effort to separate the solid chemical constituent of interest out of a mixture of inorganic powders. The inorganic powder mixture has to be insoluble and properly dispersed in aqueous media to ensure efficient GEPI recognition. In this example, the ZnO-binding peptide (VRTRDDARTHRK) identified by Kjaergaard *et al.* in 2000 (ref. 28) was used to isolate ZnO from a mixture of ZnO and Cu_2O insoluble powders.

In that aim, the lysine amino group of the ZnO-binding peptide was first bound to the epoxy group of the Dynabeads M-270 magnetic beads as shown in Fig. 1A. The GEPI ZnO (VRTRDDARTHRK) grafting to magnetic beads was visualized by optical microscopy under fluorescence, thanks to the fluorescent dye attached to GEPI ZnO *N*-terminal. Fig. 1B shows that at least one fluorescent GEPI ZnO is bound to each magnetic bead. The GEPI ZnO specificity for the ZnO powder was verified by incubating the Fluorescein-GEPI ZnO with the two separated ZnO and Cu_2O powder suspensions in the binding buffer. As seen in Fig. 1C, the GEPI ZnO peptide specifically recognizes the ZnO powder and does not bind to Cu_2O. Fig. 1C also shows that powders are partly aggregated. Fig. 1D is a further test, which proves that the peptide is indeed bound to the ZnO powder, even after washing.

Effective powder dispersion is an important parameter for the success of this inorganic particles separation process. Optimal dispersion parameters for both ZnO and Cu_2O were determined by particle size measurements by laser diffusion on insoluble powders suspension in the binding buffer. The granulometric

(A)



(B)

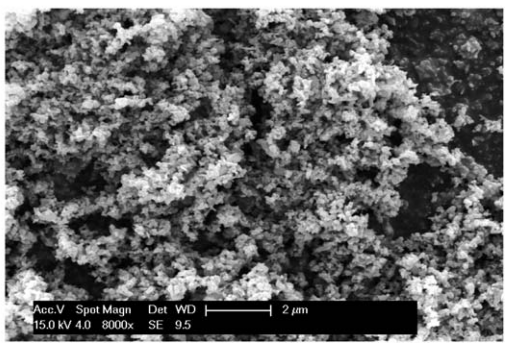


Fig. 3 Scanning electron microscopy of Cu_2O (A) and ZnO (B) powders under 8000× magnification.

curve (Fig. 2A) for ZnO powder vortexed in Tris buffer shows a broad size distribution indicating a high degree of aggregation as observed by microscopic analyses under fluorescence (Fig. 1C). As a consequence, different sonication conditions, varying powers and sonication times were tested in order to improve powder dispersion. The effect of detergent addition (Tween) on powder dispersion was also investigated. Fig. 2 shows the granulometric curves for the ZnO powder. The best dispersion condition is observed in Tris buffer pH 7.6 with 1.5% Tween 20 and after sonication at 30% for 5 minutes (Fig. 2A). Indeed, ZnO powder shows a narrow size distribution, which indicates a low degree of agglomeration. The particle size distribution lies between 0.05 and 0.16 μm which is in good agreement with the electronic microscopy analysis, which shows nanometric ZnO particles (Fig. 3B). The median particle size in number $d(0.5)$ is 0.08 μm (Fig. 2B). Concerning Cu_2O powder, the median particle size in number is 1.58 μm (Fig. 2B). The particle size is in good agreement with results obtained with electron microscopy (Fig. 3A). The stability of the powder suspension has been checked 1 h after sonication (maximum duration of one sorting experiment) and the size distribution curves remained unchanged.

The global strategy for powder separation is described in Fig. 4. First, a properly dispersed and stable ZnO–Cu_2O powder suspension is put in contact with the GEPI ZnO functionalized beads (Fig. 4A). Once in contact with the powder mixture, the ZnO insoluble powder binds to functionalized magnetic beads whereas Cu_2O powder remains in the flow through (Fig. 4B). Generally, an excess of peptide functionalized beads is incubated with ZnO and Cu_2O powder mixture (see Table 1 for the respective initial amounts) then beads are recovered by magnetisation and washed several times with binding buffer (Fig. 4C). The Zn and Cu content in pooled washing supernatants were further analysed by atomic adsorption. Finally, inorganic particles anchored to the functionalized magnetic beads were eluted and beads separated by magnetisation (Fig. 4D). The pool of eluted fractions was analysed by atomic adsorption. The Cu and Zn content of initial fractions as well as washed and eluted fractions are given in Table 1. The experiment was repeated several times with a fixed amount of beads and various amounts of ZnO and Cu_2O. Two examples of results are given in Table 1.

As shown in Table 1, the ZnO–Cu_2O mixture resulting from the GEPI-mediated sorting process is enriched in ZnO content by an 18 (experiment 1) to a 58 (experiment 2) factor as compared to the initial blend, depending on the initial amount of ZnO and Cu_2O, and this in a one step procedure. These early results document the feasibility of sorting an inorganic powder blend of various chemical compositions, using inorganic binding peptides as tools to fish out one of the constituents.

Anyway, at this stage of our developments, experimental errors on Zn and Cu quantification by atomic absorption are quite important, probably because of the scale of the experiment (volumes of suspension of max 500 μL) and the sorting process (use of magnetic beads). In fact, at each step of beads washing and elution as well as beads retrieving by magnetisation, a few μL of washing/elution fractions and a few beads may be discarded. A few beads may also be trapped in the supernatant of washing fractions and modified the ZnO quantification. By consequence, the Zn and Cu atom quantification may be altered, distorted.

In the future, sorting experiments should be performed at larger scale and various other GEPI immobilization strategies could be investigated. Instead of magnetic beads, one may use any solid support to anchor the GEPI including resin beads, fibres, tubes or membranes. The inorganic powder blend to be sorted can pass along or through the GEPI-functionalized support such that the particles of interest are captured by the inorganic-binding peptide. The support can then be removed from the powder mixture in order to fish out the particles of interest. In another approach, the inorganic-binding peptide is first contacted with the powder mixture and subsequently the complex peptide-powder of interest is attached to a support so

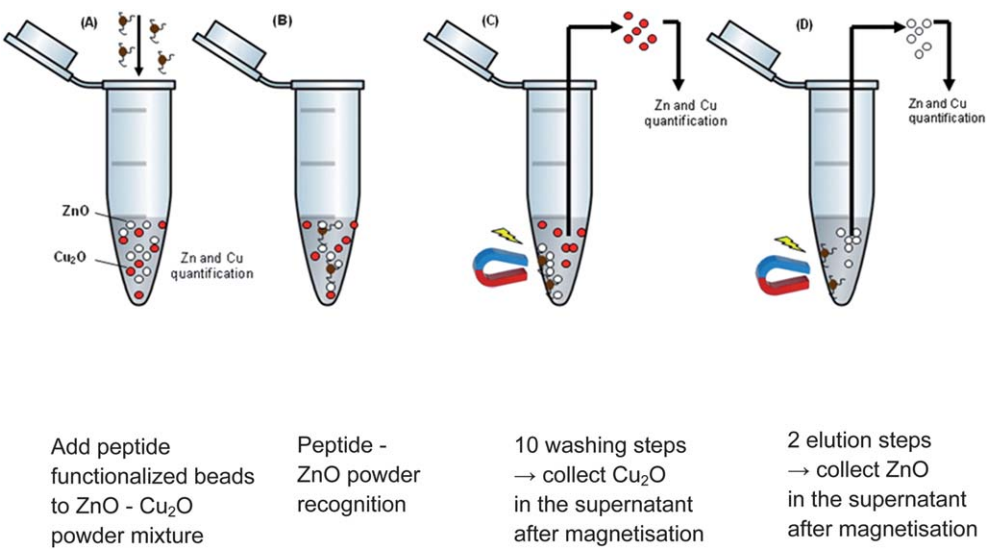


Fig. 4 Schematic representation of ZnO sorting from an insoluble ZnO–Cu_2O powders mixture. (A) GEPI ZnO functionalized magnetic beads in contact with ZnO–Cu_2O powder mixture. (B) GEPI ZnO functionalized beads recognition for ZnO powder. (C) Magnetic beads separation by magnification. (D) ZnO powder elution from magnetic beads at pH 2.2.

that it can be conveniently separated from other powder particles. The authors plan to further develop one of these sorting techniques once an interested partner/client has been identified.

4. Conclusions

In the present paper, we present a new application for inorganic-binding peptides in material science. Thanks to their high specificity for substrate chemical composition, GEPI can be used as tools to separate an insoluble inorganic nano-powder from an insoluble powders mixture of various chemical compositions. In the present paper, the complete recognition and separation process has been validated, thanks to the ZnO binding peptide grafted onto magnetic beads. The inorganic peptide mediated the interaction between the magnetic beads and the target powder. The peptide functionalized magnetic beads are put in contact with the ZnO and Cu_2O mixture and used to isolate the ZnO powder from the ZnO–Cu_2O mixture by simple magnetisation.

This technology can be expanded to the sorting of all types of inorganic insoluble particles knowing that a GEPI recognizing the powder of interest has first to be isolated by phage display. Moreover, considering GEPI specificity,[10,12] this technology could in theory be extended for isolating powders of the same chemical composition but various crystallographic forms.

Considering the advantages of the method, we believe that this process may find numerous applications in various domains where using a monodisperse powder is fundamental (pharmacy, aeronautic, microelectronic, automobile, medical, …).

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