



SDI Review Form 1.6

PART 1:

Journal Name:	International Journal of Biochemistry Research & Review
Manuscript Number:	MS: 2012/ IJBCRR/2431.
Title of the Manuscript:	Structural aspects of native and engineered metal clusters on bacterial surface layers

General guideline for Peer Review process is available in this link:

(<http://www.sciencedomain.org/page.php?id=sdi-general-editorial-policy#Peer-Review-Guideline>)

- This form has total 9 parts. Kindly note that you should use all the parts of this review form.



SDI Review Form 1.6

PART 2: Review Comments

	Reviewer's comment	Author's comment (if agreed with reviewer, correct the manuscript and highlight that part in the manuscript. It is mandatory that authors should write his/her feedback here)
<u>Compulsory</u> REVISION comments	1) Title: Misleading as the review deals NOT with structural aspects of metal clusters on S-layers! In most cases interaction of SLP with ions is described! 2) Abstract: Two third deals with SPL in general but no words concerning structural aspects of metal clusters on S-layers! 3) The authors should clearly declare which organisms have a metal uptake and which perform biomineralization, fabricate nanoclusters (and what is this) or precipitate or immobilizeions. Thi is not clear throughout the whole manuscript. 4) p. 5, line 135: What kind of binding occurred passively on the surface and how is its structure? 5) p. 6, line 156: What is the nature of this selective and reversible binding of uranium. How can it be removed and what is the structure of the bioceramic? 6) p. 8, line 194: Please write in more detail what you mean with "opens the possibilities to design templates for the synthesis of ordered metal arrays": Give some examples! 7) p. 8, line 205: Please tell us what are "supramolecular hosts and building blocks for bioelectronics devices" Give examples! 8) p. 9, line 234 to 237: The meaning of this paragraph is not clear! First the stability of the SLP is mentionend and later on that of "these nanoarrays"... 9) p. 9, section 4: Several described methods are not able to elucidate the interaction of metals with SLP. At p. 14, 4.5 no data on ions or metals are given at all.	FOR CLARITY REASONS, ANSWERS TO REVIEWER 4 ARE PROVIDED BELOW, IN A NEW PAGE.



SDI Review Form 1.6

Minor REVISION comments	1) p. 3, line 57: what are “crystalline units”? Do the authors mean “unit cell”? 2) p. 3, line 61: Please give an example for a post-translational modification of an SLP by lipids! 3) Figure 1: The quality of Figure 1 is very poor! 4) Figure 2: What are S-layer tubes. SLP recrystallized on a tube-like substrate?? 5) p. 9, line 242: which metal nanoclusters and nanomaterials can be constructed? How do they look like? 6)Figure 4: What can be seen here? On the top a stripe-like structure, but what is it? The repeating of the stripes does not fit to the lattice parameter of SbsB ... What is than the grey structure without structure? And to corrsponds the dark grey areas at the lower edges? This is not a geed image, I know much better ones even simply by looking at negative stained preparations! 7) Figure 7: Why is there a preferred binding at the boundary and not on the surface of the bacterium? Why is the control bacterium dark and the other bacterium lighter? 8) p. 19, line 539: pores are not distributed down to the subnanometer scale.	FOR CLARITY REASONS, ANSWERS TO REVIEWER 4 ARE PROVIDED BELOW IN A NEW PAGE.
<u>Optional/General</u> comments	The review reports more on the structure of SLP alone and on its interaction with metal ions without going into much detail. Structural aspects of native and engineered metal clusters on bacterial surface layers, however, got only marginal space within this review!	



SDI Review Form 1.6

ANSWERS TO REVIEWER 4

1) Title: Misleading as the review deals NOT with structural aspects of metal clusters on S-layers! In most cases interaction of SLP with ions is described!

In agreement with Reviewer 2 and 4, the title was corrected as:
Biophysical methods in the elucidation of the S-layer proteins /metal interaction.

2) Abstract: Two third deals with SPL in general but no words concerning structural aspects of metal clusters on S-layers!

As suggested by the reviewer, unnecessary descriptions were eliminated and the S-layer applications were focused to the interaction with metals and the biophysical techniques described in the manuscript.

3) The authors should clearly declare which organisms have a metal uptake and which perform biomineralization, fabricate nanoclusters (and what is this) or precipitate or immobilize ions. This is not clear throughout the whole manuscript.

According to the reviewer's request, the names of microorganisms were added when necessary (lines 105,106,114 of the revised version of the manuscript).

Most of the articles dealing with the interaction of SLPs/heavy metals have been performed on a few number of strains of extremophiles isolated from uranium mines (i.e.: *Lysinobacillus sphaericus* and *Geobacillus stearothermophilus*).

Nanocluster refers of a cluster of nanoparticles. In nanotechnology, a particle is defined as a small object that behaves as a whole unit with respect to its transport and properties. Particles are further classified according to diameter in: coarse particles (diameter within 2500-10000 nanometers); fine particles (diameter within 100-2500 nm) and ultrafine particles, or nanoparticles (diameter within 1-100 nm).

Biomineralization refers to the processes by which organisms form minerals and is now defined in line 143. A detailed explanation about the different mineralization processes can be found in reference: Weiner S, Dove PM. An Overview of biomineralization processes and the problem of the vital effect. In *Biomineralization*. Eds. P.M. Dove, S. Weiner and J.J. De Yoreo. Mineralogical Society of America, Washington, D.C., v. 54, p. 1-31. (2003).

A short explanation is given in parenthesis in lines 143 and 144 of the revised version of the manuscript.



SDI Review Form 1.6

4) p. 5, line 135: What kind of binding occurred passively on the surface and how is its structure?

The interaction of cationic metals with SLP or at least with the surface of microorganisms carrying SLP has been described as an electrostatic interaction. Carboxyl and phosphoryl groups are those directly involved in the interaction since the chemical blockage of these groups inhibit the bacteria (or SLP)/metal interaction (Halttunen T, Salminen S, Meriluoto J, Tahvonen R, Lertola K. Reversible surface binding of cadmium and lead by lactic acid and bifidobacteria. International Journal of Food Microbiology 125 (2008) 170-175). In addition, Fourier transform infrared spectroscopy results have also confirmed the electrostatic nature of the interaction (see also section 4.10, lines 514-522 of the revised manuscript).

5) p. 6, line 156: What is the nature of this selective and reversible binding of uranium. How can it be removed and what is the structure of the bioceramic?

The interaction of U with SLP has been reported to occur through phosphate and carboxyl groups. EXAFS data indicate that in whole microorganisms carrying SLP, U is coordinated by phosphate groups in monodentate bonding mode and by carboxyl groups in a bidentate fashion and these groups arise from the biocomponents of the cell surface [Merroun et al. Appl Environ Microbiol. 2005;71:5532-43 (reference 77 of the revised version of the manuscript)].

Depending on the number of available phosphate groups on the surface of the cell, the metal affinity may be higher or lower, this property being strain dependent. The high content of glutamic and aspartic acid (both residues with carboxyl groups) explains other sites of metal binding [see also ref 37,50,77 of the revised version of the manuscript].

The binding of the metals is reversible and uranium can be completely removed by using aqueous citric acid [see ref 37].

Definition of bioceramics: Bioceramics can be defined as porous filter matrices. They can be constructed by embedding cells, spores and S-layers of *B. sphaericus* in silica gels using an aqueous sol-gel process. The constructed bioceramics have an homogeneous structure and the cell biocomponents remain completely immobilized. These bioceramics are able to successfully remove copper and uranium from contaminated water [see ref 37 and also Raff J et al Bacterial-based bioremediation of uranium mining waste waters by using sol-gel ceramics. In: Merkel BJ, Planer-Friedrich C, Wolkersdorfer C, editors. Uranium in the aquatic environment. Berlin Heidelberg New York. Springer; 2002. p. 615-22].

A brief reference to the structure of bioceramics has been added in the revised version of the manuscript (line 135).

6) p. 8, line 194: Please write in more detail what you mean with “opens the possibilities to design templates for the synthesis of ordered metal arrays”: Give some examples!



SDI Review Form 1.6

Taking into account the different possibilities offered by genetic engineering techniques, design of new templates is a feasible task. This involves the insertion of aminoacid residues carrying functional groups with high affinity for heavy metals and may result in a higher efficiency for the metals sequestration. In other words, engineered recombinant SLPs enlarge the possibilities of designing nanoclusters with higher affinity and stoichiometry for metals.

In the review, we referred the insertion of cysteine or histidine residues as aminoacid residues increasing the metal binding capacity of the engineered SLPs. However, depending on the applications intended to develop, other residues may be inserted.

Note that available information about the structure of some S-layers and the use of molecular modeling tools, considerably enlarge the possibilities for the engineering S-layers with better heavy metal binding properties (see section 4.5 *In silico* structural analysis and molecular modeling).

We added a paragraph to clarify this point (lines 185-193).

7) p. 8, line 205: Please tell us what are “supramolecular hosts and building blocks for bioelectronics devices” Give examples!

Supramolecular chemistry is the domain of chemistry beyond that of molecules and focuses on the chemical systems made up of a discrete number of assembled molecular subunits or components. This domain focuses on weak and reversible noncovalent intermolecular interactions, including hydrogen bonding, metal coordination, hydrophobic forces, van der Waals forces, π - π interactions and electrostatic effects. This approach has shed light on several biological processes, including molecular self-assembly, folding, molecular recognition, host-guest chemistry, mechanically-interlocked molecular architectures, and dynamic covalent chemistry.

Considering this explanation, it becomes clear that the study of SLPs (and also the processes involving SLPs) belongs to the domain of Supramolecular Chemistry.

Taking into account that SLPs do not have intrinsically electronic and/or electrochemical functions, genetic engineering tools may modify SLPs in this direction, transforming them in potential bioelectronic devices. To clarify the idea, we rephrased our original sentence (lines 202-205).

Examples are also given in references 62-65 of the revised version of the manuscript.

8) p. 9, line 234 to 237: The meaning of this paragraph is not clear! First the stability of the SLP is mentioned and later on that of “these nanoarrays”...

This paragraph has been modified to better explain the interest of the work cited (see lines 231-236)

9) p. 9, section 4: Several described methods are not able to elucidate the interaction of metals with SLP. At p. 14, 4.5 no data on ions or metals are given at all.

We added a general paragraph defining the goal of this section: the description of some currently available methods to elucidate the interaction of SLP with metals at the beginning of section 4 (lines 255-259). To fulfil this aim, the basic information about S-layer and SLP structures is



SDI Review Form 1.6

necessary to support the analysis of SLP/metal interaction. For this reason, we first describe elementary methods to analyze basic structures of S-layers and then, how they can be used to characterize further interaction with metals.

In section 4.5, we tried to emphasize the interest of using molecular modeling for the analysis of SLPs. For instance, molecular modeling is a very useful tool for the analysis of proteins (in general). In particular, considering that working with SLPs is somehow difficult from an experimental point of view, *in silico* results may give relevant information about the architecture of SLPs and the changes induced by heavy metals. However, up to our knowledge only Horejs et al combined SLPs with molecular modeling. In their works, these authors consider that a deeper insight on the location, orientation, and steric accessibility of the SLP binding sites is crucial for the development of other applications of SLPs, including the precipitation of metal ions from solutions [Horejs et al, ref 104 of the revised version of the manuscript].

Even when the authors recognize the importance of investigations directed toward the analysis of S-layer binding sites, information about modeling of SLPs is scarce, representing a domain where much work remains to be done.

For this reason, in spite of the fact that there is no direct information about the interaction SLP/metals by molecular dynamics, the relevance of this technique deserves a special comment.

We stressed on this point in the revised version (lines 409-412 of the revised version of the manuscript).

Minor revisions

1) p. 3, line 57: what are “crystalline units”? Do the authors mean “unit cell”?

"Crystalline units" was replaced by "Unit cell".

2) p. 3, line 61: Please give an example for a post-translational modification of an SLP by lipids!

A reference about the post-translational modification of an SLP by lipids was added in the text (reference 5 of the revised version of the manuscript).

3) Figure 1: The quality of Figure 1 is very poor!

The referee is right. Figure 1 clearly loses quality when inserted in the word document.

We attach now a pdf file of this figure with a better resolution.

Note that figures loose resolution when inserted in the text. If necessary, better resolution figures are available for the final version of the manuscript.

4) Figure 2: What are S-layer tubes. SLP recrystallized on a tube-like substrate??



SDI Review Form 1.6

S-layer can recrystallize forming tubes (not on a tube-like substrate). We intended to transmit this idea in Figure 2. As indicated p. 2 lines 80-83 of the revised version of the manuscript, the S-layers directly form tubes without the need of an underlying surface.

The formation of tubes during the self assembly of S-layer proteins has been reported recently [Bobeth M, et al (ref 24 of the revised version of the manuscript)].

Only when the initial S-layer monomer concentration is in a certain range, the formation of tubes is possible [ref 24 and line 80-83].

5) p. 9, line 242: which metal nanoclusters and nanomaterials can be constructed? How do they look like?

The construction of metal nanoclusters and nanomaterials involves in many cases, microbial genetic engineering techniques to generate more metal binding sites and thus, improve the chelating capacity of SLPs. The recombinant SLPs maintain the self-assembly capacity, therefore being useful templates for the fabrication of metal nanoclusters and construction of nanomaterials, useful for different purposes (bioelectronics, biorremediation, etc). This strategy is reported for Pt and Ni in references 55-57, 73 of the revised version of the manuscript.

6) Figure 4: What can be seen here? On the top a stripe-like structure, but what is it? The repeating of the stripes does not fit to the lattice parameter of SbsB ... What is than the grey structure without structure? And to corresponds the dark grey areas at the lower edges? This is not a geed image, I know much better ones even simply by looking at negative stained preparations!

The stripe-like structure shows the crystalline structure of SbsB S-layer observed by cryoTEM, whereas the gray area corresponds to the vitrified buffer and darker gray region is the carbon of the grid. This picture has been provided by the authors of Baranova et al paper (Nature. 2012;487:119-24, ref. 44) and has been chosen to illustrate a raw image of S-layer cryo-TEM analysis where data are not processed. Several similar pictures of SbsB S-layer have been processed (Fourier Transform FFT analysis) by the authors to get the lattice parameter that can not be observed directly on this figure [please refer to figure 3 of Baranova et al (ref. 46) original publication for details].

Our main goal to show a cryo-TEM image, even if a negative staining image could look better than a cryoTEM image, is that this technique allows to observe crystals in their native state. Negative staining, (which implies dehydration, addition of staining agent and the use of high doses of electrons inducing beam damages), thus contains very limited information compared to cryo-TEM. Furthermore, the observation at -180°C under low doses conditions (less than 10 electrons/angstrom square/second) induces a better protection of the crystal than negative staining. The information that can be obtained from the analysis of a dried object molding (*i.e.* negative staining) will thus be always less than that you can get with a fresh and hydrated object (*i.e.* cryo-TEM) and this is independent of the beauty of the molding. This point has been clarified in the manuscript (line 271-273 of the revised version of the manuscript).

Caption of figure 4 has been also changed as follows:



SDI Review Form 1.6

Figure 4: Cryo-TEM image of Geobacillus stearothermophilus SbsB S-layer. The stripe-like structure corresponds to the SbsB S-layer, the gray area corresponds to the vitrified buffer and darker gray region is the plastic carbon of the grid. Image by the courtesy of R. Fronzes and G. Péhau-Arnaudet (Institut Pasteur, Paris)

7) Figure 7: Why is there a preferred binding at the boundary and not on the surface of the bacterium? Why is the control bacterium dark and the other bacterium lighter?

As this figure was not necessary for the concerned paragraph, we removed it from the new version of the manuscript.

8) p. 19, line 539: pores are not distributed down to the subnanometer scale.

The phrase was corrected.