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Development of bio-hybrid material based on *Salmonella* Typhimurium and layered double hydroxides

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The immobilization of a whole microbial cell is an important process used in nanotechnology of biosensors and other related fields, especially the development of bio-hybrid materials based on live organisms and inorganic compounds. Here, we described an essay to develop a bio-hybrid material based on *Salmonella* Typhimurium cells and layered double hydroxides (LDH). The synthetic clays have a good capacity to be a host matrix for immobilization of live entity like bacteria. The incorporation of LDH in the nutritive broth shows the capacity of bacteria to grow under the inorganic conditions. The immobilization of bacteria onto the LDH Layer deposited on gold wafers was successfully done and the verification of the final material consistence was given by Fourier transform infrared spectroscopy (FTIR) analysis that shows the possibility of various covalent links that can be established between the polar functional group of the cell and the interlayer level in the LDH. The roughness of the surface was given by scanning electron microscope (SEM) imaging and shows the homogeneity of cell distribution on the LDH layer.

Key words: Layer double hydroxide, *Salmonella* Typhimurium, Fourier transform infrared spectroscopy-attenuated total reflectance (FTIR-ATR), X-ray diffraction (XRD), energy dispersive X-ray (EDX); scanning electron microscope (SEM).

INTRODUCTION

The immobilization of enzymes for catalytic reactions coupled with redox reactions has gained an immense importance during the last two decades in the world of biosensors. Development of biosensor requires the purification of biomolecules, which is a sensitive technique and requires several critical conditions, that is

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why the investigation using these materials was left only in the research laboratories and does not rise *in situ*. Thus, the immobilization of living entities such as bacteria reduces the strokes of purification and the several conditions necessary for the bioactive layers. Bacteria can be used like bio-recognition component in biosensors to investigate the environmental toxicity caused by various media in the case of soil, sediment and water. The coupling of bacteria with device transducer can convert a cellular response into detectable signals and make it possible to exploit the sensitivity of the cell to toxins and pollutants (Hua et al., 2015). By immobilized technique, the cell can retain most of its functionality and a wide range of enzymes will be protected in the cell, which can be exploited in several applications like detection or use for the synthesis of some substances for other applications, which can then react with substrates outside the cell wall. Present as free forms, biomolecules such as enzymes, antibodies and receptors, presented in microorganisms as well as animal and plant cells or tissues have been used as biological sensing elements. Among these, microorganisms offer advantages of ability to detect a wide range of chemical substances (Banerjee et al., 2013).

Considerable attention has been given to native biomolecules in the microorganisms which conserve these biomolecules naturally and can easily modify them on large scale at the same time. It is better to keep the biomolecules solicited in their native medium which is the best method to preserve the sensitivity after the exploration of this propriety which will be seen as the ability of the whole cell to be immobilized and manipulated.

In our work, we studied the interaction between layered double hydroxides (LDHs) layer and bacterial cell. LDHs can offer various advantages such as the capability to be an interest host matrix for a guest bacteria cell and it makes it easy to study some external molecules and bacterial comportments in this new environment. LDHs can be represented by the general formula: $M^{2+}_{1-x}M^{3+}_{x}(OH)_2\ A^{n-}_{x/n}\ mH_2O$ (Ayawei et al., 2015a), M^{2+} are divalent cations (Mg^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+}), M^{3+} are trivalent cations (Al^{3+}, Cr^{3+} and Fe^{3+}) and A^{n-} is interlayer anions (Cl^-, NO^-_3 , CO^{2-}_3 and SO^{2-}_4) balancing the charge on the layers (Rezvani et al., 2014). These layers have received a great attention due to their versatile properties which are result of their high ion exchange capacity (Balcomb et al., 2015) they also have various applications such as catalysis, (Fan et al., 2014; Ayawei et al., 2015b) gene and molecular reservoir (Zhang et al., 2014) and thin films (Vlada et al., 2014).

The objective of this work was to describe the influence and interaction of low LDH on the viability of various solutions of different concentrations and the survival of *Salmonella* Typhimurium deposed on thin layer of LDH. This bio-hybrid material was checked on structural level that was confirmed by consistency of interfaces and stability of the bacteria under the LDH materials.

MATERIALS AND METHODS

Wafer device and pre-treatment

The substrate sample used in this work was 0.25 × 0.25 cm that contained a thin gold layer deposited by LPCVD (100 nm) on an Insulator/Semiconductor (IS) substrate; wafers were graciously offered by Dra Campas M., laboratory of Biosensor IRTA-Sant Carlos de la Rapita (Spain). The samples were leached with compressed nitrogen and then rinsed with MQ before being incubated for one and half hour in sulfochromic acid solution, then rinsed with water and dried with nitrogen air.

Cell culture and preparation of samples

The microbiological experiments described in this paper were carried out with *Salmonella enterica* serovar Typhimurium strain SL1344 supplied by the professor Casadesus J. (Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Seville 41080, Spain). Bacteria were incubated in nutrient broth (NB) (Pronadisa, Spain) at 37°C overnight with shaking under aerobic conditions. An exponential bacterial culture was then centrifuged at 10,000 *g* for 15 min and washed twice in sterile phosphate-buffered saline (PBS) solution and suspended in the same buffer containing LDH (at a concentration of 5, 10 and 15×10^{-3} g mL^{-1}). In parallel, control samples were prepared in PBS. Treatments were carried out at 37°C without shaking. Samples were extracted at various times (0, 3 and 6 h), diluted as needed and spread on Nutrient-agar plates then incubated for one night at 37°C. Bacteria were then counted on every plate for three repetitions, the curves of viability and survival were drawn as log (N / N_0) according to time.

Preparation of LDHs

LDHs were prepared by co-precipitation method. As per the protocol developed by Vlada et al. (2014), 50 ml of aqueous of 0.8 M $MgCl_2$ and 0.4 M $AlCl_3$ or $FeCl_3$ solution was added dropwise to 200 mL of 0.4 M sodium carbonate solution. The pH of mixture was held constant at 9.6 for MgAl-LDH and 11 for MgFe-LDH by simultaneous addition of 1 M sodium hydroxide solution (Sampieri et al., 2011). The addition of the salt solution was completed in 4 h. The precipitate was matured for 24 h at room temperature, the final solution was filtered and then washed by desionized water. The LDHs was dried at 100°C for 24 h which was followed by crushing and characterization with XRD.

Characterization technique

Powder X-ray diffraction (XRD) analysis was performed on a Panalytical X'Pert Pro diffractometer using Co K1 (40 kV, 30 mA) radiation and continued scanning mode. The diffraction patterns were recorded in a 2 theta range from 7° to 80°, in steps of 0.16° and counting time of 2 s per step.

Absorbing Infrared Fourier Transformed (IRTF) spectra in ATR mode were recorded using QUINOX 55 (Bruker) spectrophotometer in the range of 4000 – 550 cm^{1} with 2 cm^{-1} resolution and averaging 64 scans. Imaging is loaded with scanning mode using an FEI-Quanta 200 Environmental Scanning Electron Microscope.

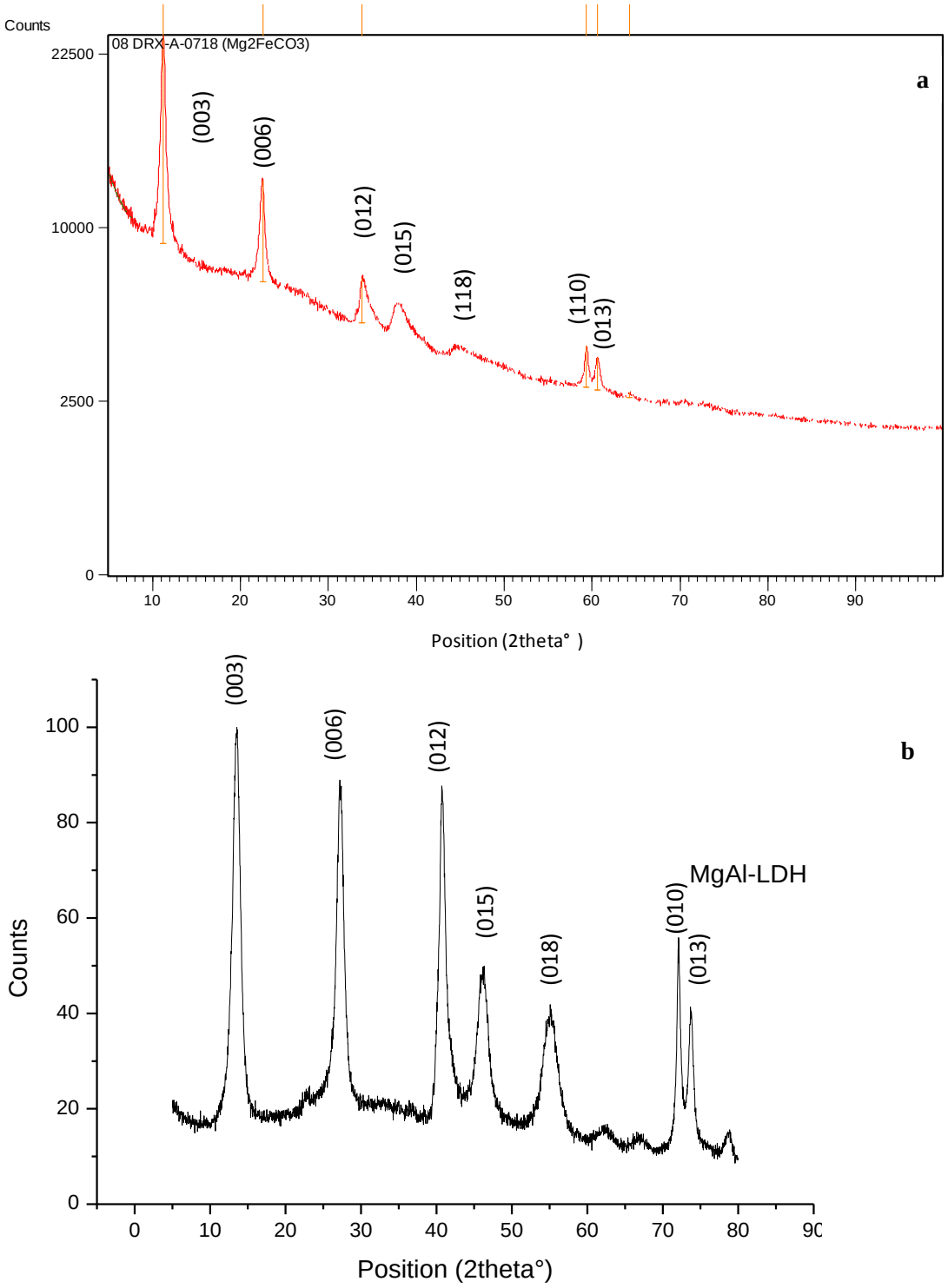


Figure 1. XR-diffractograms of Mg_4Fe (a) and Mg_2Al (b) LDHs prepared by co-precipitation.

RESULTS AND DISCUSSION

XRD characterization

The crystallinity of synthesized LDHs was analyzed with x-ray diffraction as function of weight percent inorganic component. Figure 1a and b shows x-ray diffraction pattern of various synthesized LDHs. Diffraction of $Mg_2Al(CO_3)_{0.5}(OH)_6$ *Rhomboedric R3/m* have a broad peak (006) at about $2\Theta = 25.92°$, which is a characteristic

Table 1. Comparative view based on indexed hkl plans.

hkl plans	2Θ (°) MgAl	2Θ (°) MgFe
(003)	15	12
(006)	28	23
(012)	42	34
(015)	47	38.5
(018)	55	-
(118)	-	45
(010)	72	-
(110)	-	59
(013)	75	61

Table 2. Crystalline parameters of synthesized LDHs.

Parameter	a(Å)	b(Å)	c(Å)	d(Å)
Mg_2Al	3.0	-	22.8	7.6
Mg_4Fe	3.13	3.13	15.66	-

peak of hydrotalcites (Sampieri et al., 2011; Bankauskaite and Baltakys, 2011). Studies on XRD patterns of $Mg_4Fe(OH)_{10}Cl(H_2O)3$ (Peng et al., 2014) hexagonal show all characteristic peaks of Irenty-HDLs and verifies that the crystallinity is symmetric (Dias et al., 2014).

To reveal the hkl-plans inventory of the two studied LDH, Table 1 shows many LDH characteristic peaks especially (003), (006) and (012) which are different only with the position. Many other peaks were absent and/or shifted; (018) and (010) appears respectively at 2Θ equal to 55° and 72° in the case of MgAl and does not appear in the case of MgFe, such movement in these peaks is governed by the crystalline system *Rhomboedric R3/m* in the first and *Hexagonal* in the second. However, the newest peaks appears in the case of MgFe at 45 and 59° of 2Θ position indexed to (118) and (110) respectively which can give the idea about the interlayer space and distance. To better explain the difference between the two LDH, elementary analysis have been done and the result are given in Table 2 which summarizes the crystalline parameter and confirms that the preparation of the LDH with co-precipitation synthesis method was successfully done according the study given by Hidouri et al. (2011), Abdelkader et al. (2011), Hidouri et al. (2011) and Abdelkader et al. (2011).

The value of the parameter *a* in case of MgAl was less than that of MgFe because the crystal radius of Al^{3+} was lower than that of Fe^{3+} (Chen et al., 2012; Lin et al., 2014). Because the type of M^{2+} is the same in the case of the two tested LDH, the parameter *c* value appears to be more dependent on the type of the intercalated anions CO_3 in the case of MgAl and Cl in the case of MgFe LDH.

The distinct reflections in the LDHs patterns given in Figure 1 and summarized in Table 1 indicate the formation of the typical lamellae structure and distinguish well the structure related to Al^{3+} and Fe^{3+} representing the M^{3+} of the LDH, and the intercalated ions. The details of crystalline phase given in Table 2 shows the value of the parameters of the related crystal and show the right obtained system which proved the success of the LDH synthesis using the co-precipitation method.

Capacity of bacteria to live in LDHs environment

The growth of bacteria was determined by estimation of the CFU of the suspensions at t = 0, 3 and 6 h. The analysis compared by the curves of viability of the bacterial suspensions witness and treated by synthetic clays show relative differences according to the LDH used (Figure 2). It is clear that the most important modifications got the bacterial suspensions brooded with MgAl, while the least important was recorded with MgFe.

The analysis of the curves of viability shows that the addition of the LDH practically has no considerable effect on the growth for a concentration equal to 1 and 5.10^{-3} g mL^{-1} added in the flask. However, a remarkable difference is noted for the 15×10^{-3} g mL^{-1} added in the culture; the effect becomes more noticeable for the LDH incorporate magnesium divalent metal (Figure 2a). The remarkable effect of the MgFe can take place for high concentration equal to 15×10^{-3}g mL^{-1}. The capacity of bacteria to survive in the PBS buffer doped with LDH seems more important when the concentration in LDH increases; the presence of LDHs in the medium do not have any inhibitive effect on growth of bacteria (Figure 2b), however, it appears that at this concentration the LDH has a stimulation effect in the case of 15×10^{-3} g mL^{-1} of MgAl. In the next part, bacteria was used as a model to study the relationship of *Salmonella* cells deposited on thin film of LDH, the biomaterial have been deposited on gold wafers, several techniques were used to conclude on the presence of the life entity on the surface of the inorganic compound.

The absence of inhibition of the growth of bacteria in the case of MgAl-LDH is probably due to ionization of CO_3 compound of LDH in the salt solution (Yao et al., 2015; Halma et al., 2015) that makes accessible source of carbon and enables bacteria to survive in this case. This effect is not seen in the case of LDH with MgFe because the intercalated ion was the chloride, herein the plausible hypothesis to explain this difference between LDH based on MgFe and LDH based MgAl, that MgFe is more stable and bacteria cannot use any compound of the structure which also proves that the bactericidal effect of chloride inhibits bacteria growth in this case which is comparable with the growth in the PBS solution. The conclusion relates to the hypothesis the LDH suspension conserves *Salmonella* Typhimurium growth and it depends on the intercalated ion in the LDH.

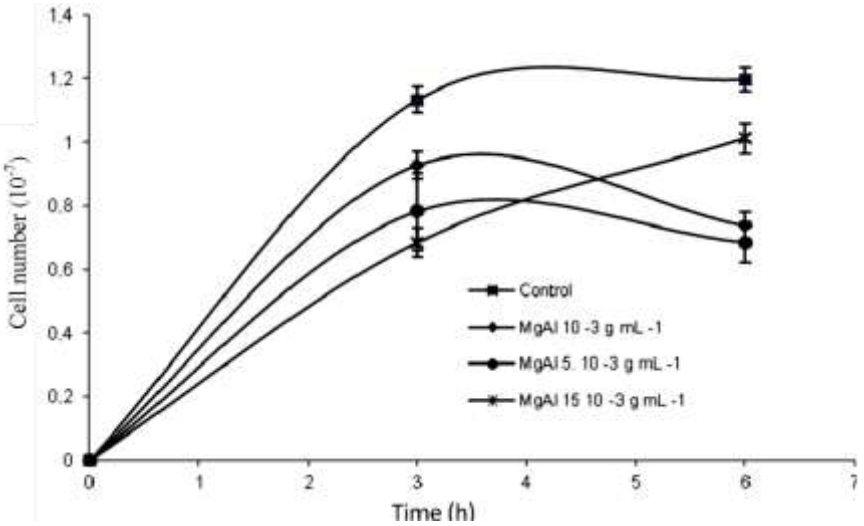


Figure 2a. Curves of viability test.

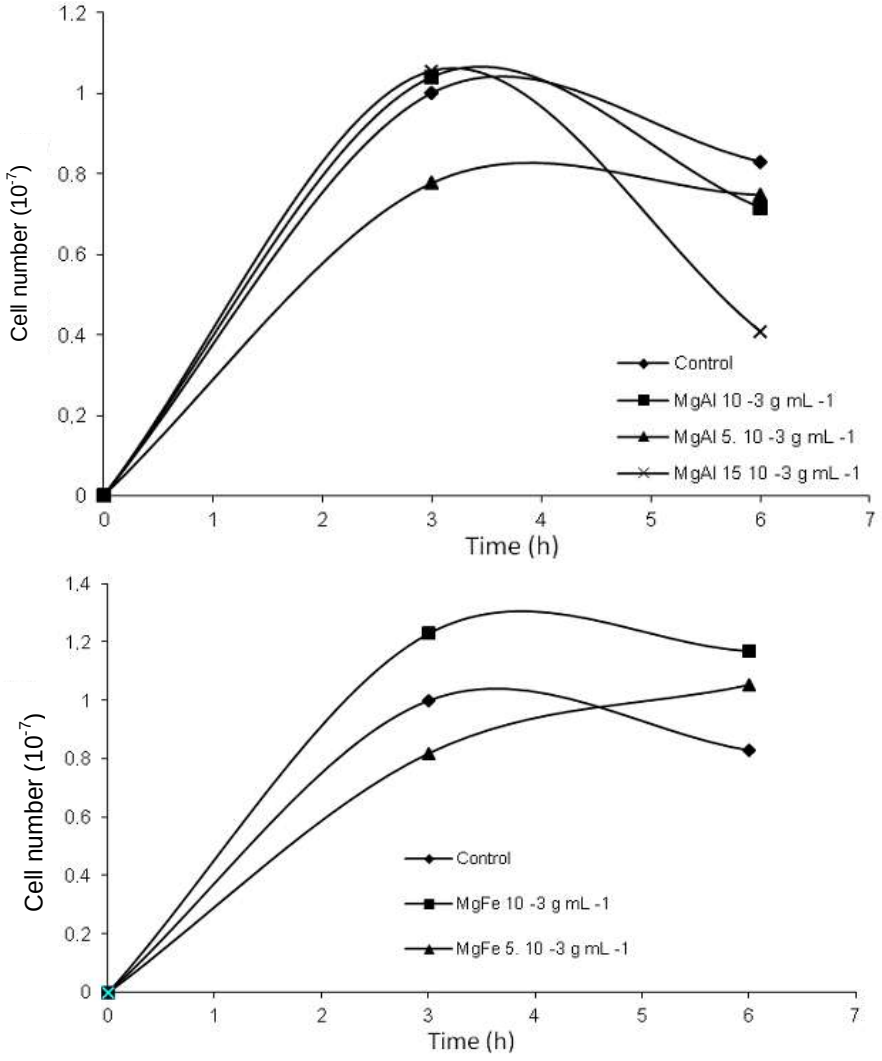


Figure 2b. Curves of viability test of bacteria in the PBS solution doped with various LDHs.

Bacterial finger on LDHs matrix

The main goal in the immobilization of bacteria is to prove that on interface level, bacteria can interact with the host matrix. FTIR analysis given in Figure 3a and b show the interface consistency in the FTIR spectrum of hybrid bacterial/LDHs/Au (surface), which were represented by the percent of transmittance and plotted as a function of wavenumber (cm^{-1}). In the case of bacteria/MgFe/Au (surface), spectra were given in Figure 3a. The spectrum exhibit various picks that demonstrate the possibility of bacteria cells to establish various types of links with MgFe-LDH, at 1564-1512 and 1485-1390 cm^{-1} which are due to the presence of N-H links. The medium intensity bond observed at 1238 and 1235 cm^{-1} assigned to $(PO_2)^-$ asymmetric stretching modes of the phosphodiester indicated that extra-membranous phospholipids can interact with LDH, also the amide III/CH2 vibration proves the role of membranous protein that react on their side, which is shown by Touisni and collaborators (2013), when the amide III/CH2 comes from the glycine backbone and protein side chain (Touisni et al., 2013). The picks situated at 1000-890 cm^{-1} are due to the =C-H bond stretching vibration. The FTIR spectra of bio-hybrid material in the case of Mg_2AlCO_3 matrix exhibit various picks distinctly which are assigned to the presence of various hydroxide links. Figure 3b summarizes the essential peaks, the thiol links at 2600-2550 cm^{-1} in both LDH with aluminum and iron which makes it possible to conclude on the role of the polar function of amino acid to participate to entrap bacteria onto LDH profound structure with her hydroxide layer.

The infrared investigation illustrate that the interactions between bacteria and HDLs was established with anions of compensators charge layer of HDLs and the free amine, hydroxide and phospholipids from bacteria. The finger of bacteria into HDL matrix was made by polar functions of membrane proteins which are in general glycoprotein. It is notable that amine groups and hydroxide of membrane proteins interacts during 12 h with the thin coat layer of HDL. The presence of carbon-carbon bonds show that amino acid carbon has also the ability to interact. The sharp bonds observed at 1656 and at 1564 cm^{-1} are assigned to the C=O stretching vibration (amide I) and to the C-N stretching/N-H bending vibration (amide II) of the tissue proteins respectively (Baccar et al., 2011). The amide bond are primarily associated with the stretching action of the C=O group. This C=O bond may be due to peptide linkage and also depends on the protein's overall secondary structure (Hussein-Al Ali et al., 2012). The various covalent links given by FTIR suggest that there is a dynamic relationship between the life entity and inert materials given here by bacteria cells and LDH.

SEM imaging of bacteria land within LDH layer

The cartography of the synthesized bio-hybrid material based on hosts LDHs and bacteria with SEM is shown in Figures 4 and 5. Scanning images were obtained from a diluted solution of bacteria. The SEM images show the presence of bacteria in LDH deposited matrix, which are homogeneously distributed throughout the layer of LDH, which is also confirmed for all samples.

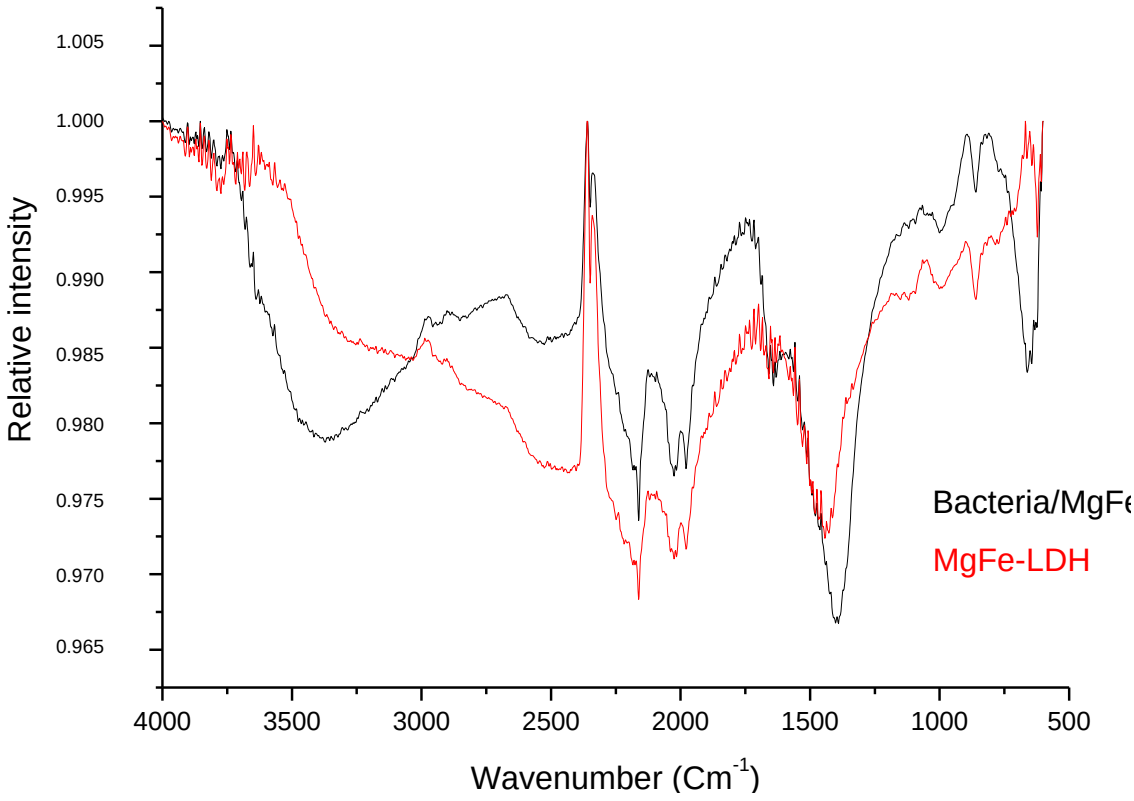
A drop deposition of viable cells of bacteria on HDLs thin layer promote oriented comportment, so that a deposited cell continues to divide and each cell produces a colony, as shown by the SEM images; there are visible areas where the density of bacteria was at the highest level, and other field still intact. It is also important to note that the area of bacteria existence confirms the results provided by the viability test; bacteria is not completely inhibited by the presence of HDL in its environment, and *Salmonella* have the ability to use HDL as a source of energy that can be carbon and/or magnesium. EDX analysis (Figure 4d and Figure 5e) confirms the chemical composition of the bacteria/LDH/Au (surface) bio-hybrid material for all samples and it was obtained at high density area of the surface. The EDX investigation show the presence of all compounds potentially present in the composite synthesized with bacteria, LDH and gold wafer.

LDH-bacteria interactions show a double capacity of *Salmonella* and LDH to be able to react with them to make one micro-hybrid material, stable and rich with various covalent links. This interaction can play an essential role in biological events, including membrane depending phenomena. In this context, SEM imaging explains various comportments of *Salmonella* with LDHs and makes clear a potentially biodegradation of LDH within *Salmonella*.

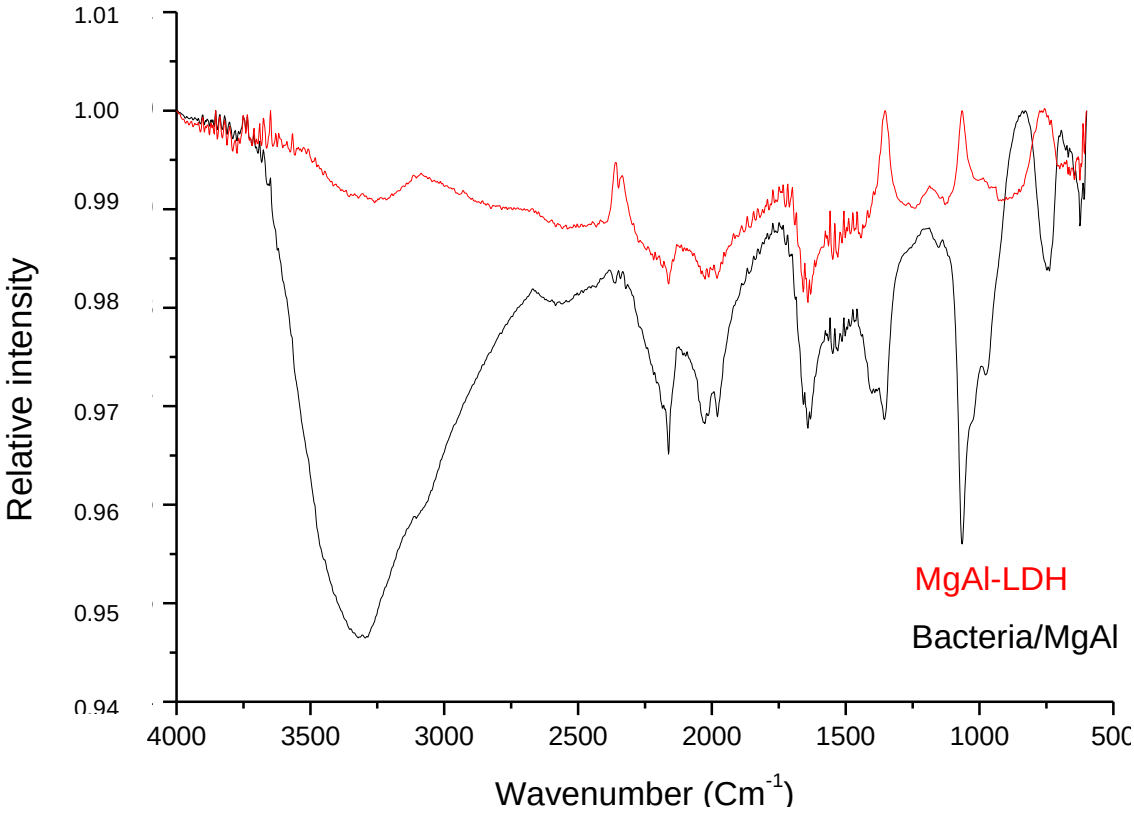
Bacteria adhesion or interaction with LDH layer is a key initiating step in nanobiotechnology based on whole cell and makes it easy to understand the process required to activate all layers that participated in the synthesized micro-hybrid materials. The interaction between *Salmonella* cells and LDH matrix makes a good profile of immobilization of the cell on inorganic compound and readjust the figure of the immobilization by inclusion. Bacteria conserves its activity, as reported by Bi et al. (2014) for biomolecules in that LDHs are a good reservoir for biomolecule for its ability to conserve the major functionality when they are immobilized on LDHs (Bi et al., 2014). It should be noted that the LDH can also be a good reservoir for bacteria cell and it conserves its biological ability and participate in bacteria cells to develop a micro-biofilm protection under LDH.

Conclusions

It is important to mention that the insignificance of differences observed in the survival of bacteria within LDHs and witness samples is given by the tolerance of *Salmonella* cells according the two tested LDH phases, MgAl and MgFe. The presence of bacterial layer in the



(a)



(b)

Figure 3. Infrared spectra of hybrid Salmonella/LDHs-Au. **(**a) Salmonella/MgFe-Au; (b) salmonella/MgAl-Au.

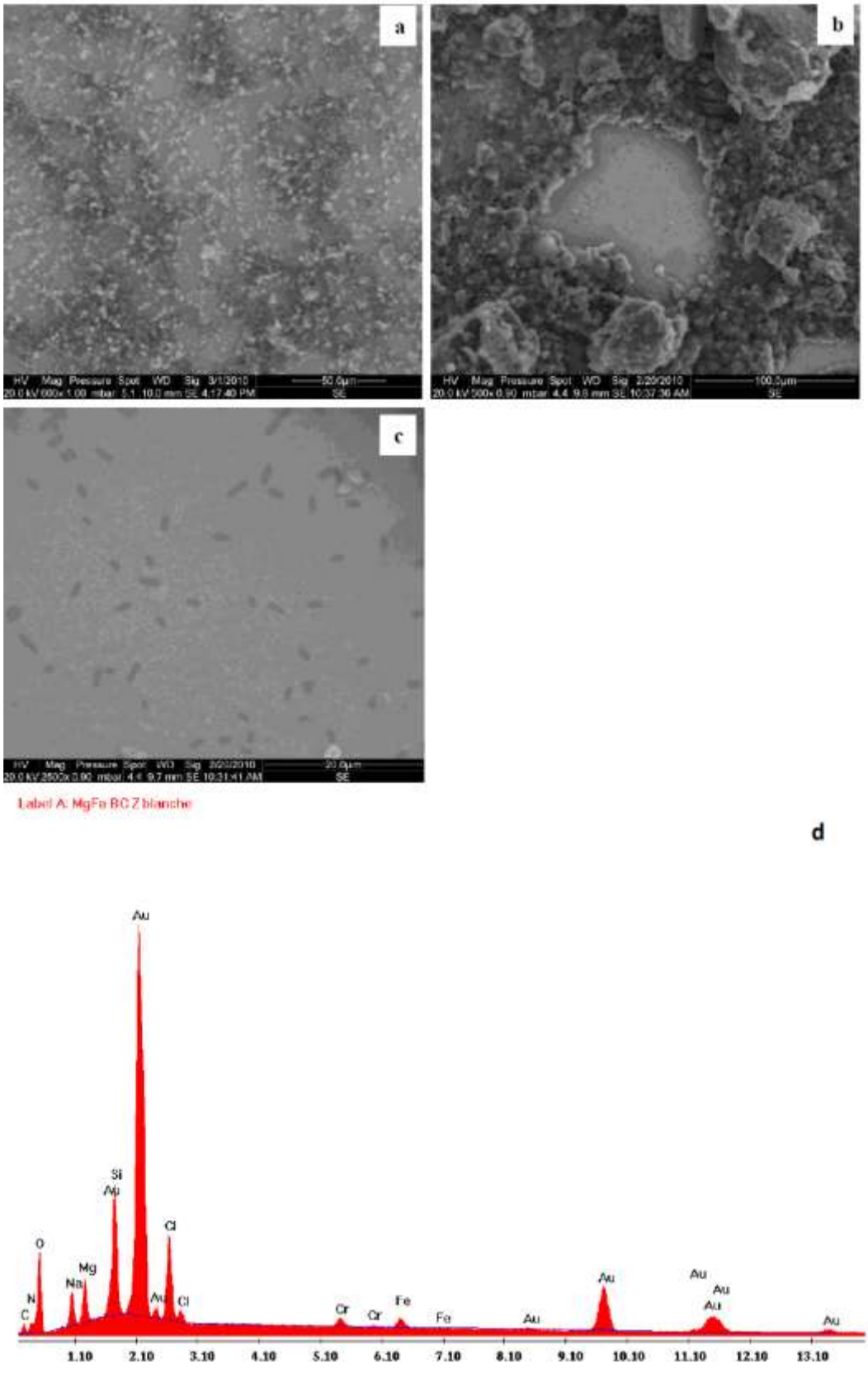


Figure 4. SEM imaging of deposited *Salmonella T*. within MgFe Layer. A. MgFe layer Scanning Image. B. Localized area with high density of bacteria within LDH layer. c. Salmonella T. in direct contact with the solid substrate of Gold. d. EDX analysis with the hybrid composite in cas of MgFe LDH.

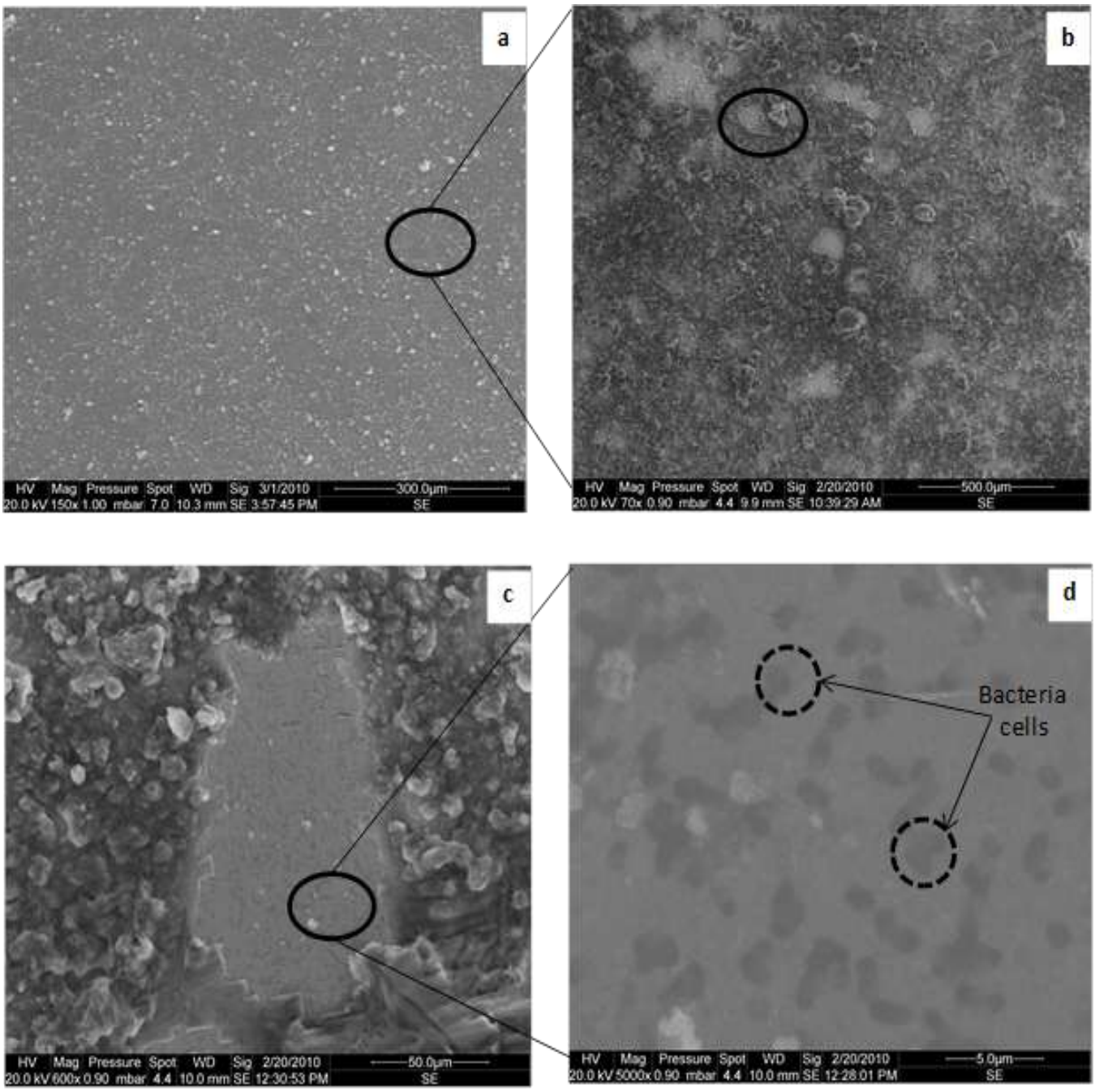


Figure 5. SEM Imaging of deposited *Salmonella* T. within MgAl Layer. (a) MgAl layer Scanning Image. (b) General vision with area of high density of bacteria within LDH layer. (c) Localized area with high density of bacteria within LDH layer. (d) *Salmonella* T. in direct contact with the solid substrate of Gold. (e) EDX analysis with the hybrid composite in the case of MgFe LDH.

integrated bio-hybrid material can offer an important advantage to biotechnology application that utilize periplasmic enzymes and more extended phospholipids and/or the extracellular polymeric substances that can catalyze reactions (Bruna et al., 2015); in this way, this bio-hybrid material can be useful for development of biosensors, the application desired in the beginning of this paper.

For the contact of bacteria cells with LDH, infrared analysis showed the presence of a wide range of possible covalent links established between LDH and viable cell of bacteria by the extra-cellular extensions like glycoproteins and phospholipids. The alteration takes place in proteins and phospholipids; it may be an adverse

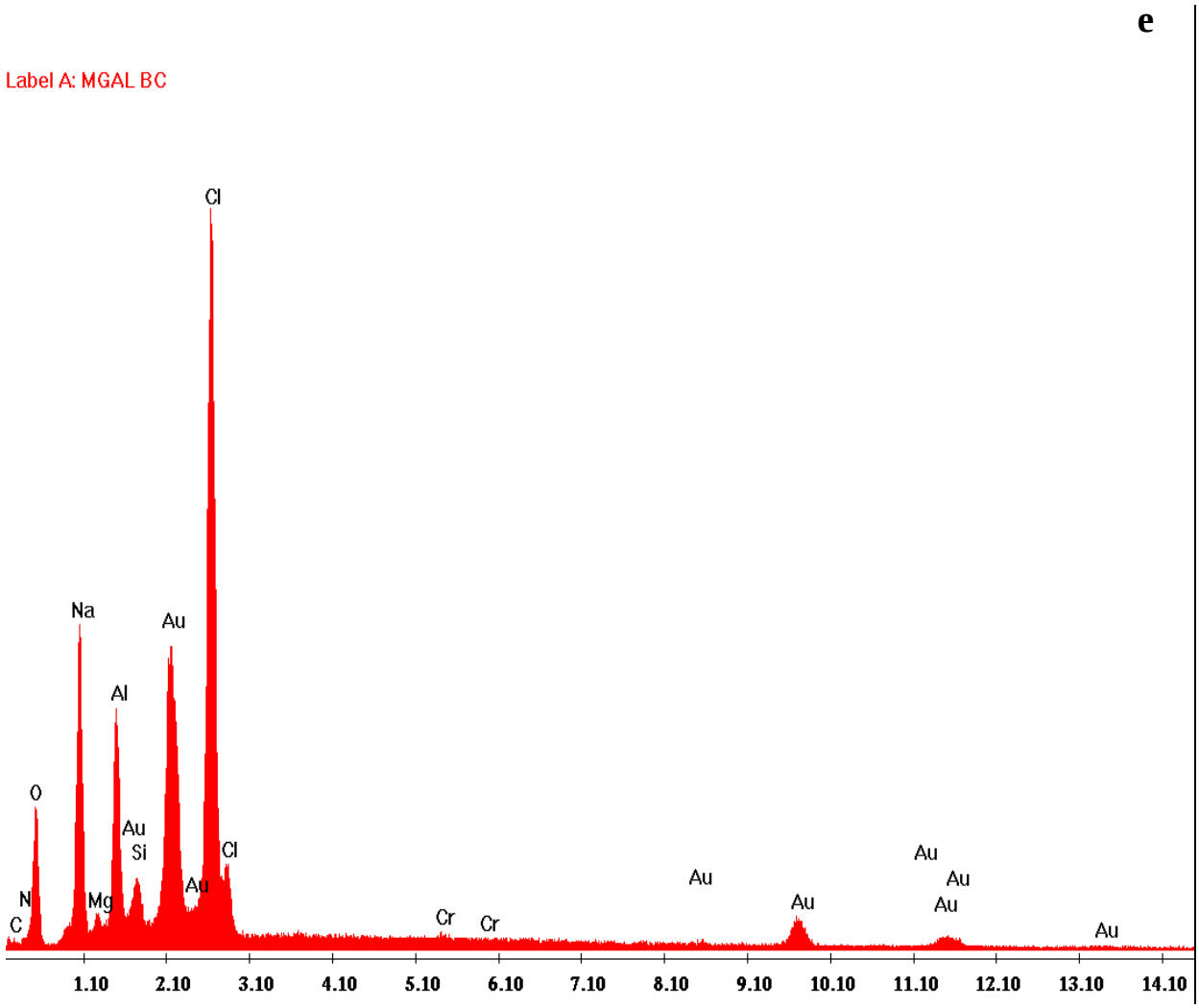


Figure 5. Contd.

effect on some important physiological processes, performing different biological events to maintain homeostasis of the cell. Therefore, the structural interactions can be considered as a good diagnostic tool carrying the effect of structural alteration caused by the covalent links on the functional processes of the bacteria. The work was done to study the feasibility of a bio-hydrid material obtained by deposition of viable *Salmonella* cells on gold wafer functionalized with LDHs and was tested at a structural side which show the good consistency and stability of the biomaterials that can be suitable for high technology applications, especially for development of biosensors and/or biopile based on immobilized live entities.

Conflict of Interests

The authors have not declared any conflict of interests.

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