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Development of an Immunosensor Based on Layered Double Hydroxides for MMR Cancer Biomarker Detection

M. Hammami*, A. Soussou, F. Idoudi, T. Cohen-Bouhacina, B. Bouhaouala-Zahar, and Z. M. Baccar

Abstract—As a potential biomarker for the investigation of cancer inflammatory profiles, macrophage mannose receptor (MMR, CD206) is herein selected to develop an immunosensor based on layered double hydroxide (LDH). Like an endocyte C-type lectin receptor, MMR plays an important role in immune homeostasis by scavenging unwanted mannose glycoproteins. It attracts a progressive attention thanks to its particularly high expression within the tumor microenvironment. There is a great of interest to develop an immunosensor based on an antibody specific to MMR for detection of stroma versus tumor cells. In this work, we studied the feasibility of high sensitive MMR cancer Screen Printed Electrode (SPE) immunosensor. Working electrode of commercialized SPE was modified by immobilization of specific antibody (anti-MMR) into thin layer of LDH nanomaterials. Structural, morphological, and surface properties of LDHs were studied by X-Ray diffraction, atomic force microscopy and Infrared spectroscopy in ATR. Cyclic Voltammetry technique was used to study interaction between the human recombinant MMR protein (rHu-MMR, NSO derived) and an immobilized antibody into developed immunosensor. High specific response of $-11.72 \mu\text{A}/\text{ng}\cdot\text{mL}^{-1}$ (with a correlation coefficient of $R^2 = 0.994$) were obtained in linear range of 0.05 ng/mL to 10.0 ng/mL of specific recombinant antigen. The limit of detection (LOD) was less than 15.0 pg/mL. From these attractive results, the feasibility of an electrochemical immunosensor for cancer was proved. Additional experiments to study stability and reproducibility the immunosensor should be completed in perspective to use these anti-MMR based immunosensors for sensing human MMR in patient biopsies and sera.

Index Terms—Immunosensor, layered double hydroxide, macrophage mannose receptor, nanomaterials.

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I. INTRODUCTION

CANCER IS THE leading cause of death in economically developed countries and the second in the leading cause of death in developing countries [1]. The accumulate evidences indicate that the development and the progression of cancer are significantly affected by interactions between the tumor cells and the stroma tumor cells as well as biomolecules and these interactions are essential for the generation of a cellular microenvironment that actively fosters tumor growth [2]–[5]. A large number of macrophages reside in the tumor stroma known as tumor associated macrophages (TAMs). These cells are mainly detected with the proliferation of tumor cells and are involved in the cell interactions mainly at the cancer metastasis level. TAMs are defined by their expression on the cell surface through antigen processing and presentation. There are two distinct profiles of TAMs depending of the level of MHC II associated complex: i) TAMs having a low MHC II expression (MHC^{low} TAMs) and ii) TAMs having a high MHC expression (MHC^{high} TAMs). The MHC^{low} TAMs are present mainly in the hypoxic area of the tumor and abundantly express the macrophage mannose receptor (MMR, CD206) whereas MHC^{high} TAMs are associated to the cancer perivascular regions [6]. In a particular tumoral microenvironment, it is well admitted that macrophages are activated and polarized in a dynamic response to combinations of cytokines to acquire specialized functional phenotypes and accordingly over expressed the MMR, an endocyte C-type lectin receptor [7] which plays a crucial role in the maintenance of the homeostasis. Hence, MMR is considered as biomarker for immunodetection and more attention is being paid to its particularly high expression in tissue pathology. Therefore, developing a highly sensitive immunosensor based on an antibody specific to MMR for detection and recording of the MMR release in the tumoral stroma as well as presentation on the surface of TAMs have a great interest. Recently, a single photon emission computed tomography (SPECT) imaging has been reported to visualize MMR on stroma cells [8]. However, this approach requires bulky equipment. It is worth noting that the antigenic performance of the incorporated antibody specific to recognize and bind MMR (anti-MMR) depends of the nanomaterial and the immobilization method used [9], [10]. Meanwhile, layered double hydroxide nanomaterials (LDHs) are considered as suitable host nanosheets for immobilizing biomolecules (i.e., enzyme, protein, and antibody) as well as many other uses [11]. LDH nanomaterial was considered as

good host matrix for antibody immobilization thanks to its bidirectional structure, high opening and anionic capacity of exchange and its large surface area [12]. Thanks to their attractive properties they are considered as suitable to promote the best conformation for metabolic reactions [13] or protein/protein interactions [14]. LDHs are known as an important class of anionic lamellar clays and could be represented by the general formula $([M_{1-x}^{II}M_x^{III}(OH)_2]_{x'} \cdot A_{x'/n}^{n-} \cdot mH_2O)$, where M^{II} are divalent cations ($Zn^{II+}, Mg^{II+}, Ni^{II+}, Cu^{II+}, \dots$), M^{III} are trivalent cations ($Al^{III+}, Fe^{III+}, Cr^{III+}, \dots$), and (A^n) are the anions ($Cl^-, NO_3^-, CO_3^{II-}, SO_4^{II-}, \dots$) intercalated into the interlayer with water molecules to compensate for positive charge of the foliar layer of LDHs structure [15]. Hence, due to their tunable composition, LDHs received a great attention for their flexibility in bidirectional nanomaterial and for their high anionic exchange capacities by changing the divalent and trivalent ratio ($x = [M^{II}]/[M^{III}]$) and by regulating the interlayer anions ($A_{x'/n}^{n-}$) to intercalate different molecules. Therefore, this kind of material can be useful for coating and functionalizing surfaces for biological detection [16]–[18]. These nanomaterials have multiple application; they can be used for monitoring food process diagnosis and medical monitoring or for therapeutic and pharmaceutical applications such as the encapsulation of active molecules for drugs [19]–[21].

II. CHALLENGE

A. Challenge

In this work, we intend to develop a highly sensitive immunosensor based on a polyclonal antibody specific to the recombinant human macrophage mannose receptor (anti-MMR) for its detection in the stroma of tumor cells.

III. METHODS AND MATERIALS

A. Chemical and Biological Reagents

The human recombinant macrophage mannose receptor (rHu-MMR) and the corresponding goat polyclonal anti-MMR were purchased from R&D systems. The recombinant human MMR peptide (rHu-MMR Leu19-Lys1383 with a C-terminal 6-His tag) is derived from NSO cell production.

NiAl-SO₄ LDHs were prepared by co-precipitation method. Briefly, 100 mL of 0.1 M NiCl₂ and 0.1 M AlCl₃ solutions were mixed drop wise under stirring conditions (1250 rpm) at 65 °C, 1h. During the mixing step, pH was maintained constant at pH = 11 by carefully adding a 2 M NaOH+0.1 M Na₂SO₄ solution. Precipitate was matured for 4 h. NiAl-SO₄ LDHs were then filtered, washed with ultra-pure water, dried at 100 °C for 24 h and crushed [17]. NiAl LDHs were used for functionalization of a commercialized gold electrode. Then, anti-MMR was encapsulated by this deposited LDHs host matrix.

B. Apparatus and Measurements

Powder x-ray diffraction (XRD) measurements were performed on a Panalytical X'Pert Pro diffractometer using CuK α

radiation ($\lambda = 1.5406 \text{ \AA}$) at 40 kV, 30 mA. The scanning rate was from 5.049° to 79.959° with a step size of 0.016°.

Fourier transform infrared (FTIR) spectra in attenuated total reflection (ATR) mode of prepared LDH thin films were recorded using VERTEX 80 V spectrometer (Bruker) in the range of 400 cm⁻¹ to 4000 cm⁻¹ with the average scan of the 32 scans.

The morphology of deposited LDHs ultra films was performed by AFM experiments in air using a Multimode setup (Nanoscope 3-Veeco-Bruker). AFM characterization was carrying out with nanosensor ppp-ncl probes in tapping mode. We choose the mica substrate as a reference because it has a flat surface with very low atomic roughness which eliminates additional contrast. The mica is added to the LDHs deposit and cleaved before each deposit to ensure a clean surface.

Gold screen printed electrodes were purchased from Dropsens company (Spain) (DRP-250 AT) with an integrated Ag pseudo-reference electrode (RE) and a Pt counter electrode (CE). The exhibited surface of the gold working electrode (WE) was 12.56 mm².

Electrochemical characterization of Biosensor was studied at room temperature, using the cyclic voltammetry measurements (CVs) of modified commercialized gold screen printed electrodes (SPEs). CV measurements were recorded using IVIUM compact stat potentiostat/galvanostat, by scanning the applied potential from -1.0 V to +1.0 V with a scan rate of 50 mV/s.

C. Preparation of rHu-MMR Specific Biosensor

The self functionalization of metal oxide film on the conducting substrate was reported in the particular case of acetylcholinesterase [18]. In fact, the structure of metal oxide materials, that is the same for layered double hydroxides (LDH), allows the functionalization of a solid substrate for any bioreceptor. The choice of materials depends on the bimolecular self assembled. NiAl-LDH metal allows the reduction of the effects of the variation in the resistivity.

rHu-MMR-specific biosensor was prepared by the modification of gold working electrode of the SPE within 2 steps, as illustrated in Fig. 1:

- Firstly, the WE of SPEs was coated by depositing a thin layer of NiAl LDHs. Precisely, a 0.25 mg of NiAl LDHs powder was dissolved in 1mL of ultrapure water (millipore), sonicated and mixed using vortex to get proper homogeneity of the sacked NiAl LDHs drop solution. Then, a 20 μ L of volume was deposited onto the gold WE and was dried at 60 °C, 20 minutes.
- The second step consists on the immobilization of the rHu-MMR biomarker: first, we deposited 200 ng of the anti-MMR dispersed in 5 μ L of physiological PBS buffer (10 mM, 0.137 M NaCl, pH = 7.4). Then, the modified electrode was dried at 37 °C, 5 min, and then at 4 °C overnight.

Finally, the unbounded antibodies were eliminated by washing modified electrode with a PBS buffer (0.1M, pH = 7.4). After each measurement, the biosensor was regenerated by immersing in 0.1 M PBS at pH 7.4 then stored physiological PBS buffer at 4 °C.

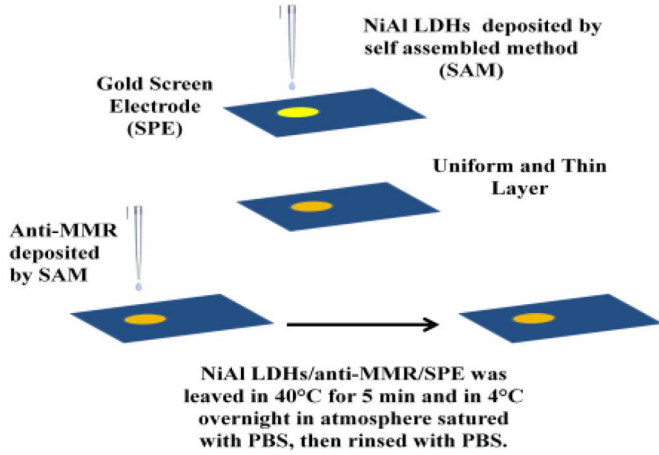


Fig. 1. Overall scheme of protocol for the preparation of NiAl LDHs/anti-MMR biomembrane.

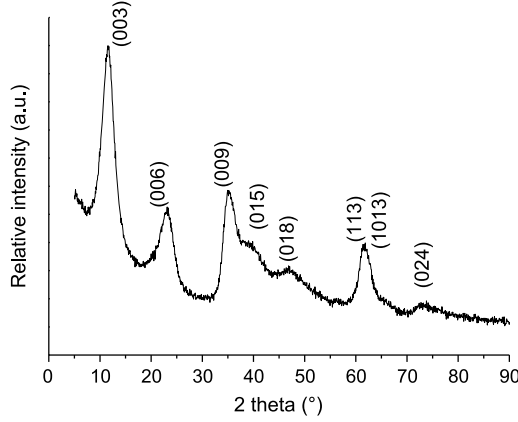


Fig. 2. XRD pattern of NiAl – SO₄ LDHs prepared by co-precipitation method.

IV. RESULTS AND DISCUSSION

A. NiAl LDHs Characterization

1) *Structural Characterization of LDH Powder:* The XRD pattern shown in Fig. 2 confirms the crystalline phase identity of prepared NiAl LDH. The shape and the symmetrical peaks are in agreement with those reported in literature [18]. The diffraction peaks at 11.2°, 23.15°, 35.2°, 39.8, 47°, 57.1°, 62.179°, and 73.8° are ascribed to the (003), (006), (009), (015), (018), (113), (1013), and (024) plane of typical hydrotalcite like NiAl-LDH. The basal spacing around 0.75 nm calculated from (003) planes indicates the intercalation of CO₃²⁻ ions into the interlayer spaces.

The average crystallite size of nanoparticles was calculated using Debye-Scherrer ((1)).

$$D = \frac{K\lambda}{\beta \cos\theta} \quad (1)$$

where K is a constant equal to 0.9, λ is the wavelength of CuK α radiation, β is the full width at half maximum (FWHM) of the diffraction peak in radiant and θ is the bragg angle of the main planes. The average crystallite size of NiAl-LDH is 3.5 nm.

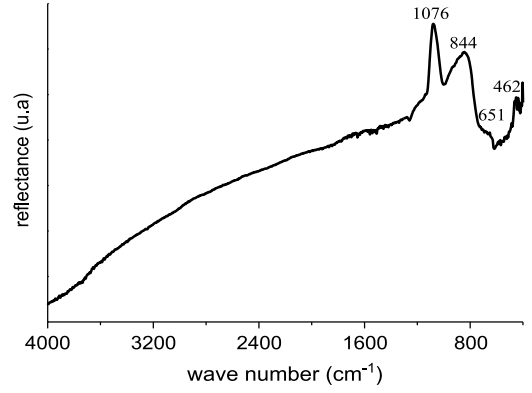


Fig. 3. FTIR spectra of NiAl LDHs.

The identification of the pattern using ICDD database indicated a molar ratio of Ni: Al cations of [3:1] and the crystal group system was rhombohedral R3/m.

2) *Infrared Study of LDH Thin Films:* A 20 μ L of 0.25 mg/mL of NiAl-LDHs solution was deposited on the silica substrate (the exhibited surface was approximately 12 mm² and dried for 20 min at 60 °C.

FTIR with reflectance attenuated total reflection (ATR) mode has been proven to be feasible technique to obtain spectroscopic features regarding thin films [22], [23].

Fig. 3 shows the FTIR spectra of NiAl LDHs. The bands around 1076 cm⁻¹ and 844 cm⁻¹ showed the presence of interlayer carbonate anions [24], [25]. Furthermore, the bands around 651 cm⁻¹ and 462 cm⁻¹ are ascribed to the characteristic peaks of Ni-OH and Al-OH vibrations in the lattice of LDHs. As shown in Fig. 2, almost all the characteristic peaks are observed in the spectrum of NiAl LDHs.

3) *Morphological Study of LDHs Nanofilms:* Different concentrations of LDHs solutions (ranging from 0.25 mg/mL to 2.50 mg/mL) were tested by self-assembly deposition to optimize the choice of the adequate concentration. Before antibody immobilization, a drop of LDH solution (approximately 15 μ L by cm²) was deposited on the mica substrate and was allowed to dry for 20 min to 25 min. The solvent was evaporated in air at room temperature allowing the film to self-assemble on the substrate surface.

The sample surface was then rinsed with ultrapure water to eliminate non-bonded nanoparticles before AFM characterization.

Topographic AFM images shown in Fig. 4 present typical results obtained in the study of the concentration effect on NiAl LDH films when deposited by self-assembly on the mica [26], [27].

These observations indicate that the deposited LDH films are in the form of homogeneous layer (brown/dark areas) with aggregates (bright areas) of different sizes, more or fewer, depending on the used concentrations.

The brown/dark areas correspond to a homogeneous organization of LDH film. A scratching experience [28] performed routinely on the deposited film allowed us to verify that, indeed, the mica reference substrate is covered completely by the film (results not shown).

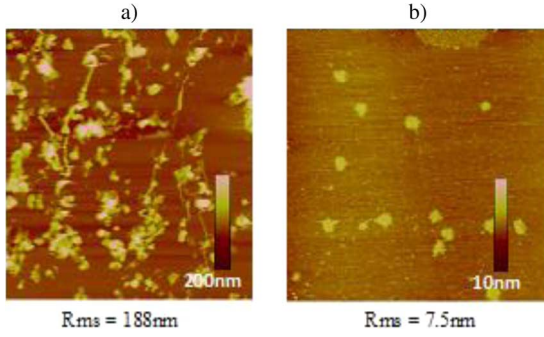


Fig. 4. Topographic AFM images ($20\ \mu\text{m} \times 20\ \mu\text{m}$) obtained in tapping mode: Concentration effect on NiAl LDH films deposited by self assembly on the mica. The Rms and C parameters are the average surface roughness and the NiAl concentrations respectively.

TABLE I
THE AVERAGE ROUGHNESS ASSOCIATED WITH ALL TESTED CONCENTRATIONS EXTRACTED FROM TOPOGRAPHIC AFM IMAGES (SCANS SIZE OF $10\ \mu\text{m} \times 10\ \mu\text{m}$)

NiAl LDH concentrations (mg/mL)	2.5	1.5	0.75	0.5	0.25
The average surface roughness Rms (nm)	250	123	7	~7.5	6

For high LDH concentrations (from 1.5 to 2.5 mg/mL), the obtained films were thick and heterogeneous containing stacked aggregates and relatively high roughness estimated to 188 nm (Fig. 4(a)). Whereas the surfaces of NiAl LDH films obtained from lower concentration's solutions, were more homogenous and tidy (Fig. 4(b)). The decrease in the concentration allows reducing in the number and size of aggregates of LDH nanoparticles.

Table I includes the average roughness associated with all tested concentrations. We clearly see that the average surface roughness continues to decrease when decreasing concentration: for concentrations lower than 0.75 mg/mL, the obtained layers of LDH were homogeneous and flat and the roughness was estimated at 6 nm –7 nm. Therefore, for coating gold electrode by ultrathin layer of LDH, the chosen concentration value was 0.25 mg/mL.

B. rHu-MMR-Specific MMR Biosensor Characterization

The electrochemical studies of prepared Hu-MMR immunosensor was carried out at room temperature by scanning 10 successive cycles from $-1.0\ \text{V}$ to $1.0\ \text{V}$ with a rate scan of $50.0\ \text{mV/s}$ CVs in physiological PBS buffer with $59.18\ \text{mM}$ of iron cyanide redox couple.

Fig. 5 shows the CVs scanned with non modified and modified gold working electrode. The cyclic voltammograms of the bare SPE (curve a) exhibited a reversible redox reaction and a particular behavior due to the high electron transfer between electrolyte and the bare SPE. Then, a reduction of the average of the redox potential and a shift of the oxidation peaks to $200\ \text{mV}$ were recorded. This is due to the high capacity of ion exchange of the LDH. Finally, no significant change in shape of CVs after immobilization of the anti-MMR into the modified gold electrode surface (curve c) was noticed. However, the reduction

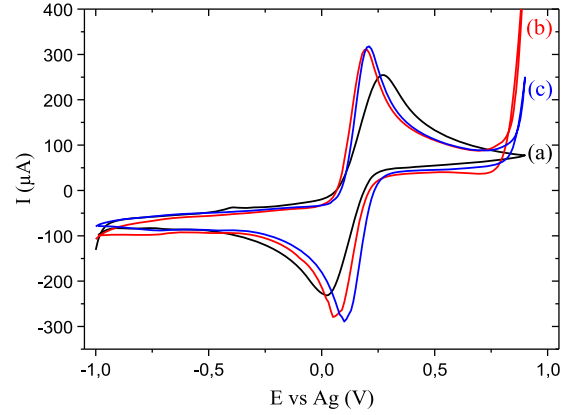


Fig. 5. Cyclic voltammogram comparison of screen printed electrode immunosensor before and after surface treatment: (a) initial SPE before modification; (b) after deposition of LDH; (c) after immobilization of anti-MMR onto LDH.

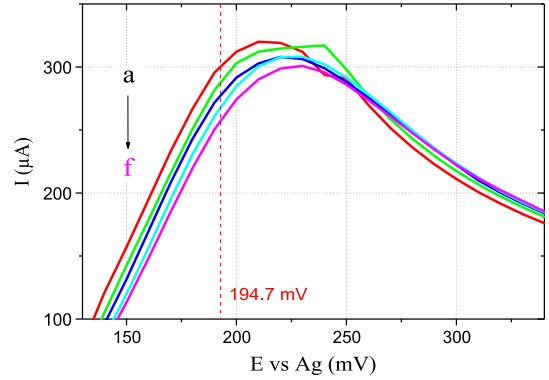


Fig. 6. Oxidation peaks of cyclic voltammograms of NiAlLDH/anti-MMR modified SPE immunosensor after rHu-MMR biomarker injection at different concentrations: (a) 0 ng/mL; (b) 0.05 ng/mL; (c) 2.5 ng/mL; (d) 12.5 ng/mL; (e) 15 ng/mL; (f) 20 ng/mL.

peak shift indicates the successful of mass transfer of rHu-MMR and the deposition of an ultra-thin layer of the biomarker.

The immunosensor response were studied by adding rHu-MMR biomarker concentrations from initial physiological PBS buffer (blank: 0) to 20 ng/mL. Upon the addition of the rHu-MMR biomarker, the magnitude of oxidation current peak (at $200\ \text{mV}$) decreases vs antigen concentration. This confirms the detection of interaction between rHu-MMR and the anti-MMR/NiAl-LDH/SPE. Moreover, the high anodic current peak obviously can be explained by the signification role of LDH in the electrocatalytic oxidation of the mannose.

In fact, the diffusion of the substrate molecules towards the metal sites inside the LDH (where the OH^- diffusion-migration from the solution to the surface of LDH) oxidized the $\text{Ni}^{\text{II}+}/\text{Ni}^{\text{III}+}$, producing an electron transfer charge complex [29]. The oxidation of the rHu-MMR by the oxido-reductive NiAl-LDH increased charge transfer into the sensor.



The sensitivity of the immunosensor was investigated and the best responses were obtained at the applied potential of 194.7

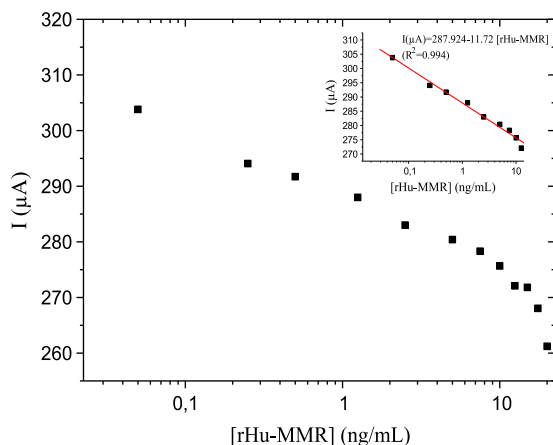


Fig. 7. Calibration curves of the immunosensor for successive addition of rHu-MMR targeting physiological PBS at room temperature and with an applied potential of 194.7 mV.

mV. Fig. 7 represents the obtained calibration curves of the immunosensor for successive addition of rHu-MMR. The obtained curves exhibit two linear regressions for lower concentration than 10.0 ng/mL and for upper concentrations.

The first linear regression was related to the specific interaction between the immobilized antibody onto the sensor and the target molecule (rHu-MMR). As shown in the inset of Fig. 7, the sensitivity of the immunosensor was $-11.72 \mu\text{A}/\text{ng}\cdot\text{mL}^{-1}$ (with a correlation coefficient of $R^2 = 0.994$) in the linear range of 0.05 ng/mL to 10.0 ng/mL. The limit of detection, estimated by extrapolation, was less than 15 pg/mL.

However, for concentration of anti-MMR higher than 10.0 ng/mL, the slope increase to $-51.8 \mu\text{A}/\text{ng}\cdot\text{mL}^{-1}$ with a correlation coefficient of $R^2 = 0.81$. This kinetic reaction changes could be due to the detection of both Non specific and/or specific (antiMMR/rHu-MMR) interactions that takes place at the Electrolyte/Biosensor junction.

V. CONCLUSION

In this work, we have demonstrated the feasibility of LDH-based method for development of a high sensitive immunosensor for the detection of tumoral stroma biomarkers as the macrophage mannose receptor presented by tumor associated macrophages (TAMs) in cell cancer microenvironment.

The method exhibited in this paper for coating and functionalizing could be applied for any commercialized antigen and was very easy to implement. The specific interaction between the immobilized antibody (anti-MMR) onto modified low cost electrodes (SPEs) and the rHu-MMR so called CD206 or MRC1 have been demonstrated for concentrations lower than 10.0 ng/mL and the limit of detection was estimated to be lower than 15 pg/mL. This very low value of limit of detection can be useful in early stage diagnosis of cancer diseases.

REFERENCES

[1] M. Xioma and Y. Herbert, "Global burden of cancer," *Yale J. Biol. Med.*, vol. 79, no. 3–4, pp. 85–94, Dec. 2006.
 [2] B. Engels, D. A. Roweley, and H. Schreider, "Tragetting stroma to treat cancer," *NIH Semin. Cancer Biol.*, vol. 22, no. 1, pp. 41–49, Feb. 2012.

[3] S. Kim, H. Takahashi, W.-W. Lin, P. Descargues, S. Geivennika, Y. Kim, J.-L. Luo, and M. Karin, "Carcinoma produced factors activate myeloid cells via TLR 2 to stimulate metasis," *NIH Nature*, vol. 457, no. 7225, pp. 102–106, Jun. 2009.
 [4] R. N. Kaplan, R. D. Riba, S. Zacharoulis, A. H. Bromley, L. Vincent, C. Costa, D. D. Macdonald, D. K. Jin, K. Shido, S. A. Kerns, Z. Zhu, D. Hicklin, Y. Wu, J. L. Port, N. Altorki, E. R. Port, D. Ruggiero, S. V. Shmelkov, K. K. Jensen, S. Rfi, and D. Lyden, "VEGRA1-positive hematopoietic bone marrow progenitors initiate the pre-metastatic niche," *NIH Nature*, vol. 438, no. 7069, pp. 820–827, Dec. 2005.
 [5] W.-W. Lin and M. Karin, "A cytokine-mediated link between innate immunity, inflammation, cancer," *JCI Clin. Invest.*, vol. 117, no. 5, pp. 1175–1183, May 2007.
 [6] S. A. Linehan, L. M. Pomares, R. P. d. Siliva, and S. Gorden, "Endogenous ligands of carbohydrate recognition domains of the mannose receptor in murine macrophages, endothelial cells and secretory cells potential relevance," *Eur. J. Immunol.*, vol. 31, no. 6, pp. 1857–1866, May 2001.
 [7] K. Movahedi, D. Laoui, C. Gysemans, M. Baeten, G. Strangé, J. V. d. Bossche, M. Mack, D. Pipleers, P. I. Veld, P. D. Baetselier, and J. A. V. Ginderachter, "Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C (high) monocyte," *Cancer Res.*, vol. 70, no. 14, pp. 5728–5739, Jul. 2010.
 [8] K. Movahedi, S. Schoonoghe, D. Laoui, I. Houbracken, W. Waelput, K. Breckpot, L. Bouwens, T. Lahoute, P. De Baetselier, G. Raes, N. Devoogdt, and J. A. Van Ginderachter, "Nanobody-based targeting of the macrophage mannose receptor for effective *in vivo* imaging of tumor-associated macrophages," *Cancer Res.*, vol. 72, no. 16, pp. 4165–4177, Jun. 2012.
 [9] V. Puddu and C. C. Perry, "Interaction at the silica-peptide interface: The influence of particule size and surface fonctionality," *Langmuir*, vol. 30, no. 1, pp. 227–233, Dec. 2013.
 [10] B. C. C. Pessela, G. D. Ortiz, L. Betancor, M. Fuentes, J. M. Guissan, and R. F. Lafuente, "Modulation of the catalytic properties of multimeric β -galactosidase from E.Coli by using different immobilization protocols," *Enzyme Microb. Technol.*, vol. 40, no. 2, pp. 310–315, Jan. 2007.
 [11] D. G. Evans and W. Duan, "Preparation of layered double hydroxides and their applications as additives in polymers, as precursors to magnetic materials and in biology and medicine," *Chem. Commun.*, vol. 7, pp. 485–496, Oct. 2005.
 [12] Z. M. Baccar, S. Hidouri, N. El Bari, N. Jaffrezic, A. Errachid, and N. Zine, "Stable immobilization of anti-Beta casein antibody onto layered double hydroxides materials for biosensor applications," *Sensor Lett.*, vol. 7, pp. 676–682, Nov. 2009.
 [13] M. Temani, Z. M. Baccar, and H. B. Mansour, "Activity of cholesterol oxidase immobilization hydroxide nanomaetrial for biosensor application: Acacia salicina scavenging power of hypercholesterolemia therapy," *Microelectron. Eng.*, vol. 126, pp. 165–168, Jul. 2014.
 [14] Z. M. Baccar, N. Zine, N. J. Renault, and A. Errachid, "Development of urease/layered double hydroxides nanohybrid materials for the urea detection, synthesis, analytic and catalytic characterizations," *Sensor Lett.*, vol. 7, pp. 647–655, Nov. 2009.
 [15] P. Beaudot, M. E. D. Roy, and J. P. Besse, "Preparation characterization of intercalation compounds of Layered double hydroxide with metallic oxalato complexes," *Chem. Mater.*, vol. 16, no. 5, pp. 935–945, Feb. 2004.
 [16] Z. M. Baccar, D. Caballero, R. Eritja, and A. Errachid, "Development of an impedimetric DNA-biosensor based on layered double hydroxide for the detection of long ssDNA sequences," *Electrochim. Acta*, vol. 74, pp. 123–129, Apr. 2012.
 [17] Z. M. Baccar, S. Hidouri, A. Errachid, and O. R. Sanchez, "Study of bi-enzyme immobilization onto layered double hydroxides biosensor application," *Nanosci. Nanotechnol.*, vol. 11, pp. 8798–8803, Oct. 2011.
 [18] S. Hidouri, Z. M. Baccar, H. Abdelmelek, T. Noguier, J.-L. Marly, and M. Compas, "Structural and functional characterization of a hybrid material based on layered double hydroxides," *Talanta*, vol. 85, pp. 1882–1887, Jun. 2011.
 [19] J. K. Park, Y. B. Choy, J.-M. Oh, J. Y. Kim, S.-J. Hwang, and J.-H. Choy, "Controlled release of donepezil intercalated in smectite clays," *Int. J. Pharm.*, vol. 359, no. 1–2, pp. 198–204, Jul. 2008.
 [20] M.-M. Hong, J.-M. Oh, and J.-H. Choy, "Encapsulation of flavor molecules 4-hydroxy-3-methoxy benzoic acid into layered inorganic nanoparticles for controlled release of flavor," *Nanosci. Nanotechnol.*, vol. 8, pp. 5018–5021, Oct. 2008.

- [21] M. Back, J.-M. Choy, and S.-J. Choi, "Montmorillite intercalated with glutathione for antioxidant delivery synthesis, characterization and bioavailability evaluation," *Int. J. Pharm.*, vol. 425, no. 1–2, pp. 29–34, Apr. 2012.
- [22] Q. Tao, J. Zhu, R. L. Frost, T. E. Bostrom, R. M. Wellard, J. Wei, P. Yuan, and H. He, "Silylation of layered double hydroxides via a Calcination-Rehydration Route," *Langmuir*, vol. 26, no. 4, pp. 2769–2773, 2009.
- [23] M. Milosevic, S. L. Berets, and S. L. Fadeev, "Single-reflection attenuated total reflection of organic monolayer on silicon," *A. Y. Appl. Spectrosc.*, vol. 57, no. 6, pp. 724–727, 2003.
- [24] V. Rives and S. Kannan, "Layered double hydroxides with the hydrotalcite-type structure containing Cu²⁺, Ni²⁺ and Al³⁺," *J. Mater. Chem.*, vol. 10, no. 2, pp. 489–495, Jan. 2000.
- [25] A. Vald, R. Birjega, A. Matej, C. Luculescu, B. Mitu, M. Dinescu, R. Zavoianu, and O. D. Pavel, "Retention of heavy metals on layered double hydroxides thin films deposited by pulsed laser deposition," *Appl. Surface Sci.*, vol. 302, pp. 99–144, 2014.
- [26] W. Cantrell and G. E. Ewing, "Thin film water on muscovite mica," *J. Phys. Chem.*, vol. 105, pp. 5434–5439, 2001, B.
- [27] K. El Kirat, I. Burton, and V. Dupres & Y. F. Dufrene, "Sample preparation procedures for biological atomic force microscopy," *J. Microsc.*, vol. 218, pp. 199–207, 2005.
- [28] N. R. Faye, F. Moroté, C. Grauby-Heywang, and T. Cohen-Bouhacina, "Oxydation of langmuir-blodgett films of monounsaturated lipids studied by atomic force microscopy," *Int. J. Nanotechnol.*, vol. 10, no. 5/6/7, p. 390-40, 2013.
- [29] E. Scavetta, D. Tonelli, M. Giorgetti, F. Nobili, R. Marassi, and M. Berrettoni, "AC impedance study of a synthetic hydrotalcite-like compound modified electrode in aqueous solution," *Electrochim. Acta*, vol. 48, pp. 1347–1355, 2003.