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Platelet-derived extracellular vesicle functionalization of Ti implants

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TITLE:

Platelet-Derived Extracellular Vesicle Functionalization of Ti Implants

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SUMMARY:

Here, we present a method for the isolation of Extracellular Vesicles (EVs) derived from the platelet lysates (PL) and their use for coating titanium (Ti) implant surfaces. We describe the drop casting coating method, the EVs release profile from the surfaces, and *in vitro* biocompatibility of EVs coated Ti surfaces.

ABSTRACT:

Extracellular Vesicles (EVs) are biological nanovesicles that play a key role in cell communication. Their content includes active biomolecules such as proteins and nucleic acids, which present great potential in regenerative medicine. More recently, EVs derived from Platelet Lysate (PL) have shown an osteogenic capability comparable to PL. Besides, biomaterials are frequently used in orthopedics or dental restoration. Here, we provide a method to functionalize Ti surfaces with PL-derived EVs in order to improve their osteogenic properties.

EVs are isolated from PL by size exclusion chromatography, and afterward Ti surfaces are functionalized with PL-EVs by drop casting. Functionalization is proven by EVs release and its biocompatibility by the lactate dehydrogenase (LDH) release assay.

INTRODUCTION:

EVs are membrane vesicles (30–200 nm) secreted by any cell and play a key role in cell-to-cell communication by delivering their cargo. They contain a variety of active biomolecules that may include nucleic acids, growth factors, or bioactive lipids[1]. For these reasons, EVs have been evaluated for their potential use in therapeutics. In terms of orthopedics and bone regeneration, EVs from different sources have been tested. Among them, platelet-derived EVs have been shown to induce a differentiation effect on stem cells while maintaining a low cytotoxic profile[2,3]. Therefore, further research is required to explore the possibility of combining EVs with biomaterials in order to use them in daily clinical practice.

Titanium-based biomaterials are widely used as scaffolds for bone healing clinical interventions due to their mechanical properties, high biocompatibility, and long-term durability[4]. Nevertheless, Ti implants are a bioinert material and, therefore, present a poor capability for bonding with the surrounding bone tissue[5]. For this reason, titanium modifications are being studied in order to improve their performance by achieving a more functional microenvironment on its surface[4,6,7]. In this sense, EVs can be anchored to titanium by chemical[8] or physical interactions[9,10]. Immobilized EVs derived from stem cells or macrophages enhance the bioactivity of Ti by promoting cellular adhesion and proliferation thereby inducing an osteogenic effect[8–10].

This article will focus on a drop casting strategy for coating Ti surfaces with PL-derived EVs in detail. In addition, we will evaluate EVs release profile from the coated surface over time and confirm its cellular biocompatibility *in vitro*.

PROTOCOL:

Platelet Lysate (PL) is obtained as previously described in compliance with institutional guidelines[3] using fresh buffy coats provided by the IdISBa Biobank as starting material. Their use for the current project was approved by its Ethics Committee (IB 1995/12 BIO).

1. EVs isolation from PL

1.1. Larger bodies removal

1.1.1. Thaw PL at room temperature.

1.1.2. Centrifuge PL at 1,500 x *g* for 15 min at 4 ˚C. Discard the pellet as it contains cell debris.

1.1.3. Collect the supernatant and perform two consecutive centrifugations at 10,000 x *g* for 30 min at 4 ˚C.

NOTE: The pellet corresponds to larger EVs such as microvesicles, and in this case, it is discarded.

1.1.4. Filter the supernatant first through 0.8 µm porous membrane, and then through 0.2 µm porous membrane.

NOTE: These steps remove all non-desired EVs.

1.1.5. Pool the filtered PL and store at -20 ˚C until use.

1.2. Size exclusion chromatography

1.2.1. Equilibrate the column coupled to chromatography equipment at the desired flow rate with filtered PBS.

NOTE: The flow rate used depends on the column characteristics; in this case, it is set to 0.5 mL/min.

1.2.2. Load the processed PL (5 mL) with a syringe to the equipment.

1.2.3. Inject the PL into the column and start collecting 5 mL fractions in 15 mL tubes.

1.2.4. Collect the EVs enriched fractions and store them at -80 ˚C until use.

NOTE: When performing the experiment for the first time, characterize all fractions by protein quantification and immunodetection to determine the one enriched with EVs[3,11]. In this experiment, the 9th fraction is collected.

1.2.5. Wash the chromatographic column with 30 mL of 0.2% NaOH solution and store it in 20% ethanol solution once it reaches equilibrium.

[Place **Figure 1** here]

2. EVs characterization

NOTE: EVs characterization is necessary to perform functional studies[12]. Electron microscopy or western blot characterization have previously been reported[13]. This report will focus on the essential characterization techniques for Ti surface functionalization.

2.1. Nanoparticle Tracking Analysis (NTA)

2.1.1. Dilute the EVs (1:1000) in 0.2 µm filtered PBS.

NOTE: Too concentrated samples or too diluted samples will be out of range for NTA determination, and adjustment will be required.

2.1.2. Load 1 mL of the diluted EVs with a syringe to the NTA equipment and inject them into the NTA equipment.

2.2.3. Follow the manufacturer's protocol for particle concentration and size distribution determination.

2.2. Protein concentration

2.2.1. Determine the concentration using 1 µL of the EVs solution. Measure the absorbance with a spectrophotometer at a wavelength of 280 nm.

NOTE: EVs should present low levels of proteins compared to the number of particles.

2.2.2. Follow the manufacturer's instructions to obtain the absorbance reading using the spectrophotometer.

3. Titanium surface functionalization

NOTE: In this method, machined titanium discs, c.p. grade IV, 6.2 mm diameter, and 2 mm height, are used. The discs may be manipulated with Ti tweezers, but it is important not to scratch the surface. Moreover, the machined side must face upwards during the entire process.

3.1. Ti discs wash

NOTE: The volume of solutions used for Ti washing should be enough to cover Ti discs. Place Ti discs in a glass beaker and pour solutions onto them. Then, remove the solution by decanting.

3.1.1. Wash Ti implants with deionized (DI) water, and then discard the water.

3.1.2. Wash Ti implants with ethanol 70%, and then decant to remove the solution.

3.1.3. Place the implants in DI water and sonicate at 50 ˚C for 5 min. Discard the water.

3.1.4. Incubate Ti implants in a 40% NaOH solution at 50 ˚C for 10 min with agitation. Discard the solution.

CAUTION: NaOH solution warms during preparation. The solution is corrosive and should be used inside a fume hood.

3.1.5. Sonicate the implants in DI water at 50 ˚C for 5 min, and then remove the water.

3.1.6. Perform several washes with DI water (at least 5) until it reaches to neutral pH. Check pH with pH indicators.

3.1.7. Sonicate the implants in DI water at 50 ˚C for 5 min and remove the water.

3.1.8. Incubate Ti implants in a 50% HNO_3 solution at 50 ˚C for 10 min with agitation. Remove

the solution.

CAUTION: HNO_3 is a corrosive and oxidizer substance, and it should be used inside a fume hood.

3.1.9. Sonicate the implants in DI water at 50 ˚C for 5 min. Remove the water.

3.1.10. Perform several washes with DI water (at least 5) until neutral pH is obtained. Check the pH with pH indicators.

3.1.11. Sonicate the implants in DI water at 50 ˚C for 5 min. Remove the water.

NOTE: At this point, the experiment can be stopped by storing Ti implants in a 70% ethanol solution.

3.2. Ti passivation

NOTE: Ti passivation steps are performed by completely covering Ti discs with the different solutions in the order listed below. Ti discs are placed in a glass beaker and solutions are gently poured on them. Volumes used in all wash steps must completely cover the implants and are removed via decanting.

3.2.1. Incubate the Ti implants in a 30% HNO_3 solution for 30 min at room temperature under gentle agitation. Remove the solution.

3.2.2. Perform several washes with DI water (at least 5) until it reaches to neutral pH. Check the pH with pH indicators.

3.2.3. Incubate Ti implants overnight at room temperature in DI water.

3.2.4. Dry off the implants under vacuum conditions at 40 ˚C for 10 min.

3.3. EVs drop casting

NOTE: For cell functional studies, it is important to work in a cell culture cabinet.

3.3.1. Place the Ti implants in a 96-well plate, with the machined side facing up.

NOTE: If the implants are turned upside-down, a needle can be used to set them back.

3.3.2. Thaw the EVs solution and mix them with agitation. Use a vortex to pulse for 3 s.

3.3.3. Deposit the EVs on the Ti surface. In this study, drops of 40 µL of EVs solution are placed onto the Ti to immobilize a maximum of 4×10^{11} EVs per implant according to the concentration determined by NTA.

3.3.4. Place the plates containing the Ti under vacuum conditions at 37 ˚C until drops are completely dry (~2 h).

NOTE: Adjust the time depending on the number of implants and the water present in the vacuum chamber.

[Place **Figure 2** here]

4. Ti surface characterization

4.1. Release study

4.1.1. Incubate Ti surface with 200 µL of filtered PBS at 37 ˚C.

NOTE: PBS is filtered to avoid interferences with the NTA measurement.

4.1.2. Replace the PBS at different time points and store at -80 ˚C.

NOTE: In this study, 2-, 6-, 10-, and 14-days' time points were analyzed.

4.1.3. Analyze stored PBS for particle studies by NTA according to the manufacturer's instructions.

NOTE: Particle concentration in PBS at different times is a representation of EVs release profile over time.

4.2. Biocompatibility studies

NOTE: Human umbilical cord-derived mesenchymal stem cells (hUC-MSC) are obtained from the IdISBa Biobank in compliance with institutional guidelines.

4.2.1. Maintain hUC-MSC in DMEM low glucose supplemented with 20% FBS until use. Change the medium twice per week.

4.2.2. For cell seeding, wash the cells in flasks with 5 mL of PBS twice.

4.2.3. Trypsinize hUC-MSC by adding 1 mL of trypsin solution. Ensure that it completely covers the monolayer of cells. Remove the trypsin solution and place the cell culture flask at 37 ˚C for 2 min approximately. View cell detachment under the microscope. Detached cells will appear round in shape and will be in suspension.

4.2.4. Resuspend cells in DMEM low glucose with 1% EVs depleted FBS.

NOTE: Prepare media supplemented with 1% FBS, and then ultracentrifuge at 120,000 x *g* for 18 h to remove FBS-EVs. It is important to remove EVs to avoid interferences with platelet EVs.

4.2.5. Determine cell concentration by counting the number of cells with a Neubauer chamber[14].

4.2.6. Bring hUC-MSC to a concentration of 50,000 cells/mL.

4.2.7. Seed 200 µL of the cell solution onto the Ti implants.

4.2.8. After 48 h, collect 50 µL of media and perform the cytotoxic determination using lactate dehydrogenase (LDH) activity kit, according to the manufacturer's protocol.

REPRESENTATIVE RESULTS:

The method presented in this article allows obtaining EVs functionalized titanium discs. EVs are physically bonded to the surface, which allows a sustained release over time. The amount of EVs released can be measured by NTA on Day 2, 6, 10, and 14. The first measurements, on Day 2, show that around 10^9 EVs are released, followed by a sustained release on day 6 (~10^8 EVs); day 10 (~10^7 EVs), and day 14 (~10^7 EVs). This confirms a sustained release, despite showing a decrease in the amount of EVs released over time.

[Place **Figure 3** here]

Moreover, biocompatibility studies performed on MSC reveal that after 48 h of cell growth onto Ti and Ti-EVs, an improvement in biocompatibility was observed in Ti-EVs compared to the Ti control group, shown by the lower LDH activity levels of the Ti-EV group compared to the Ti group. Media was collected after 48 h of cell growth on implants. Cells grown directly on tissue culture plastic were used as a negative control with 0% LDH activity, while cells treated with 1% Triton X-100 were used as a positive control with 100% cytotoxicity.

[Place **Figure 4** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic diagram of Platelet Lysate (PL) extracellular vesicle (EVs) isolation. PL is centrifuged first at 1,500 x *g*, and then at 10,000 x *g* to remove larger bodies. The supernatant is filtered through 0.8 and 0.2 µm filters. Processed PL is loaded onto the column, and EVs are separated by size exclusion chromatography.

Figure 2: Schematic diagram of Ti passivation and EVs functionalization by drop casting. Ti implants are passivated first by incubation for 30 min in a 30% HNO_3 solution at room temperature. After several washes with DI water, pH reaches neutral. Then, Ti implants are incubated overnight at room temperature in DI water. After that, the implants are dried off under

vacuum conditions at 40 ˚C. For EVs immobilization, 40 µL of EVs solution are deposited onto Ti implants. Next, implants are incubated at vacuum for 2 h until EVs are physically bound to the surface.

Figure 3: Accumulative EV release of Ti functionalized surfaces. Particles were released in PBS on days 2, 6, 10, and 14 at 37 ˚C. Data represents the mean ± SEM with n = 3.

Figure 4: *In vitro* cell biocompatibility of Ti-EVs. LDH activity was measured in culture media 48 h after cell seeding onto the implants. Cells seeded on tissue culture plastic were set as 0% of toxicity while cells seeded onto tissue culture plastic and treated with triton X-100 1% were set as 100% of toxicity. A dashed line is shown at 30%, which is the maximum value accepted for cytotoxicity of medical devices according to ISO-10993:5. Data represent the mean ± SEM, with n = 15 (three independent experiments were performed).

DISCUSSION:

This protocol aims to provide clear instructions for EVs functionalization onto Ti surfaces. The method presented is based on a drop casting strategy, which is a physisorption type of functionalization. Poor bibliography exists regarding EVs functionalization on Ti surfaces, although there are few studies showing different advantages by immobilizing EVs on Ti[10]. Anyway, some of the strategies explored include biochemical binding[8], polymeric entrapment[9] or drop casting[10]. Though the use of chemical coatings through covalent bonds might achieve a more homogeneous coating with a higher grade of functionalization, chemical reactions may harm the EVs structure and functionality[15]. Drop casting is an easy and low-cost method compared with polymeric entrapment or biochemical binding.

One important point of the protocol that can be addressed is the EV source. In this study, EVs are obtained from PL. However, the drop casting method is adaptable to any kind of EVs, since it is based on physical interactions. Previous studies with other methods present positive results after evaluating the use of cell cultured EVs such as stem cells[8,10] or machropages[9]. It is important, regarding the use of EVs, to perform a complete characterization of them. The International Society of Extracellular Vesicles aims to determine most of the main EVs parameters in order to assure reproducibility in the field[12]. Other studies have already described the methodology for EVs characterization, thus in this protocol we have not detailed the electronic microscopy and western blot technique protocols performed[13].

A critical step for Ti functionalization by drop casting is the time and conditions allowed for EVs physisorption. In the protocol we present, incubation under vacuum conditions is performed until drops are completely drought. Usually, 2 h are needed to assure water evaporation at 37 °C and under vacuum conditions. However, the number of implants being functionalized may increase the time needed to ensure the correct adhesion of EVs on Ti. It is important to make sure that no water is left before proceeding to characterization or functional studies. However, variations in the protocol for EVs functionalization onto Ti can be found in the literature. For instance, an overnight incubation at 4 ˚C without vacuum conditions has already been explored[10]. However, in our hands, the use of this method led to poor results compared to the complete dry

that we describe.

Though not performed in the present protocol, EVs functionalization might be evaluated through different methodologies, among others, changes on the surface wettability might be characterized by measuring the water contact angle on the surface; and changes in the chemical nature of the coatings by Fourier-transformed infrared (FTIR) spectroscopy coupled to optical microscopy. Moreover, EVs might be stained with specific dyes (such as PKH26 dye), and the functionalized surfaces might be imaged by fluorescence microscopy.

Overall, further functional tests can be performed to explore the osteogenic functionality of EVs deposition on Ti. On the one hand, cell adhesion or growth assays performed through confocal microscopy or enzymatic activity can be the first approach to test functionality[10]. In this paper, we have described the cytotoxicity assay as one of the first approaches of implants' effects on cells. On the other hand, PCR assays can be used to determine the gene expression of osteogenic markers in cell culture performed on Ti discs[8–10]. Moreover, protein detection through western blot can also suggest an osteogenic profile, despite being a semi quantitative technique. Other protein detection techniques such as detection arrays or enzymatic kits could also be good approaches for functionality experiments' performance[9]. An additional functional assay is the determination of calcium deposition through Calcein Blue Staining[16]. Once *in vitro* functionality has been proved, further experiments could be performed using animal moldels[8].

In conclusion, surface functionalization allows an improved therapeutic design of biomaterials. The combination of EVs with implantable biomaterials can allow sustained release associated to an improvement of biocompatibility and osteogenic properties of the biomaterial. It is important to explore different approaches for EV binding; thus, drop casting is an interesting starting point for future studies that aim to produce clinically available orthopedical devices. The protocol presented in this manuscript aims to give an easy and reproducible guide for future experiments' performance.

ACKNOWLEDGMENTS:
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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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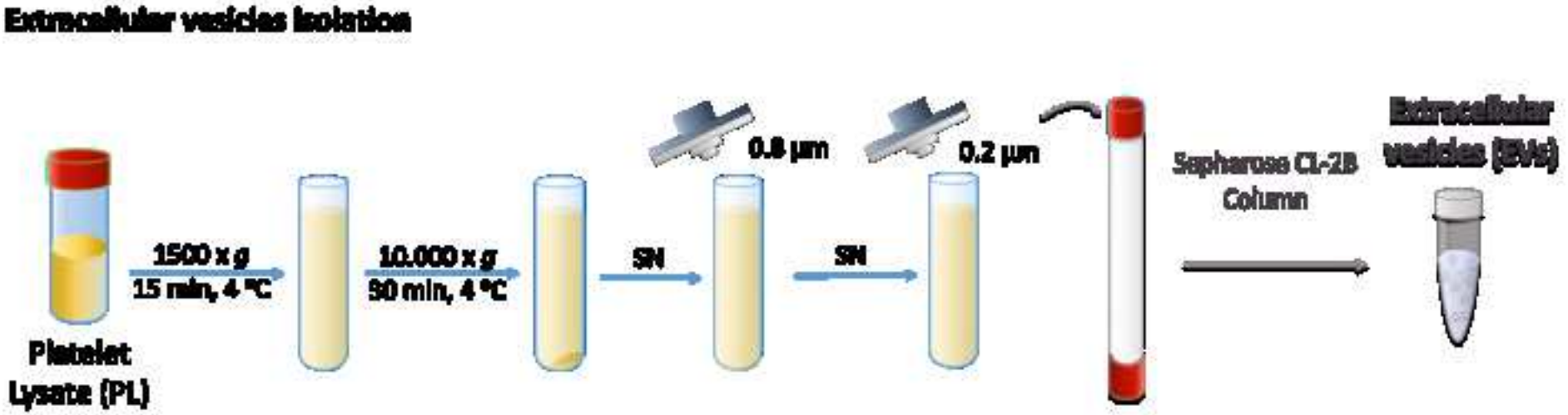


Figure 2

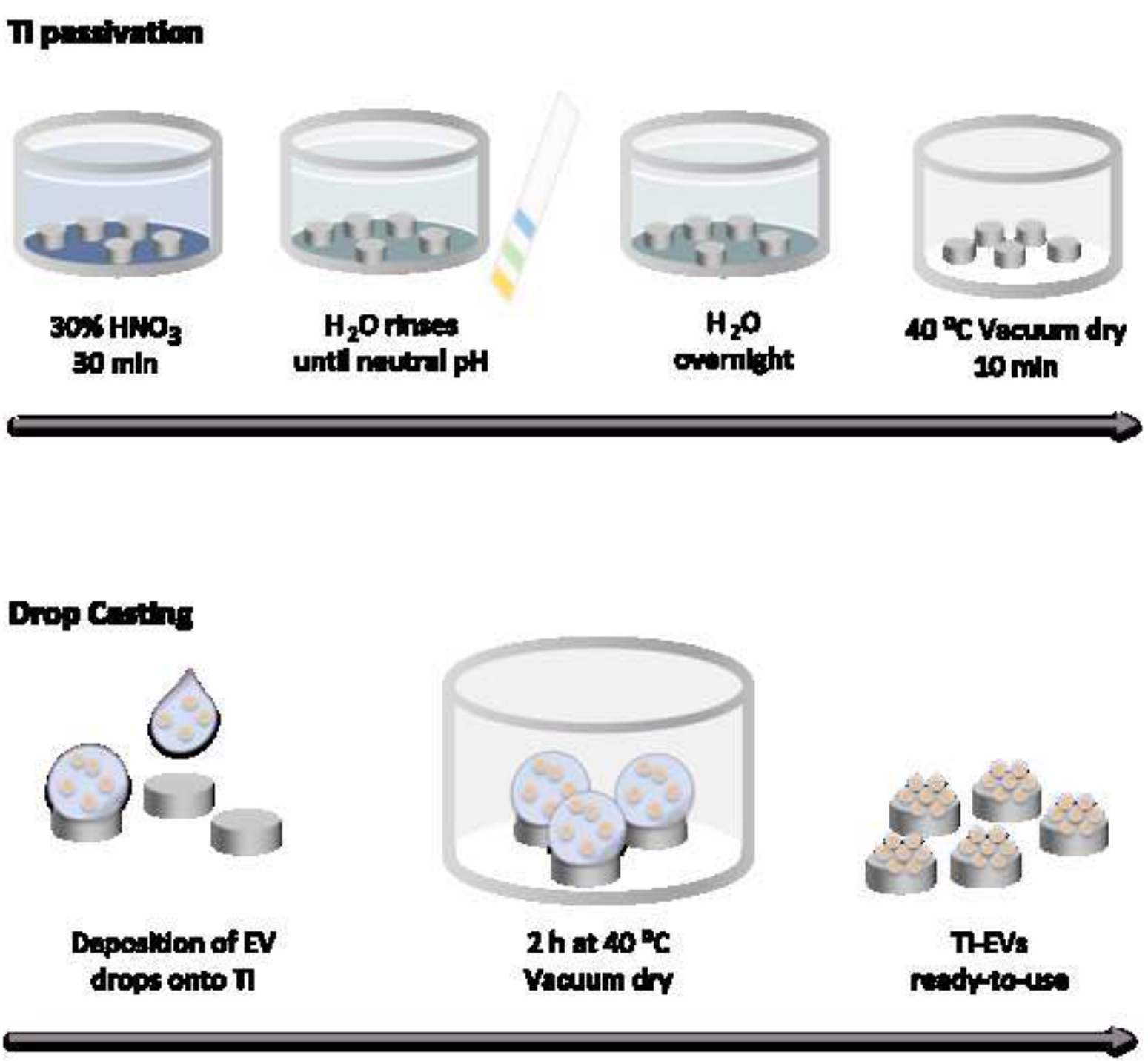


Figure 3

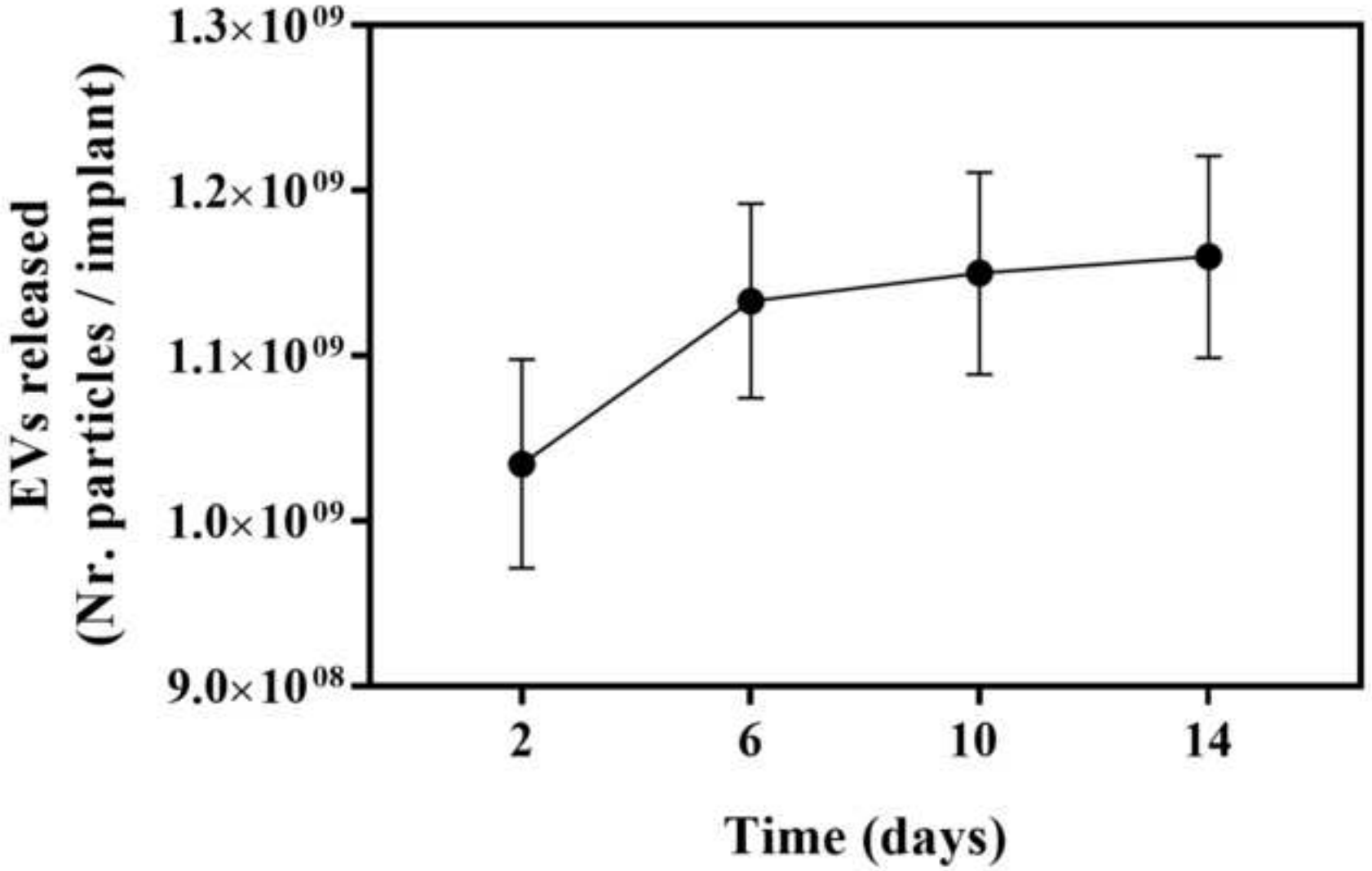
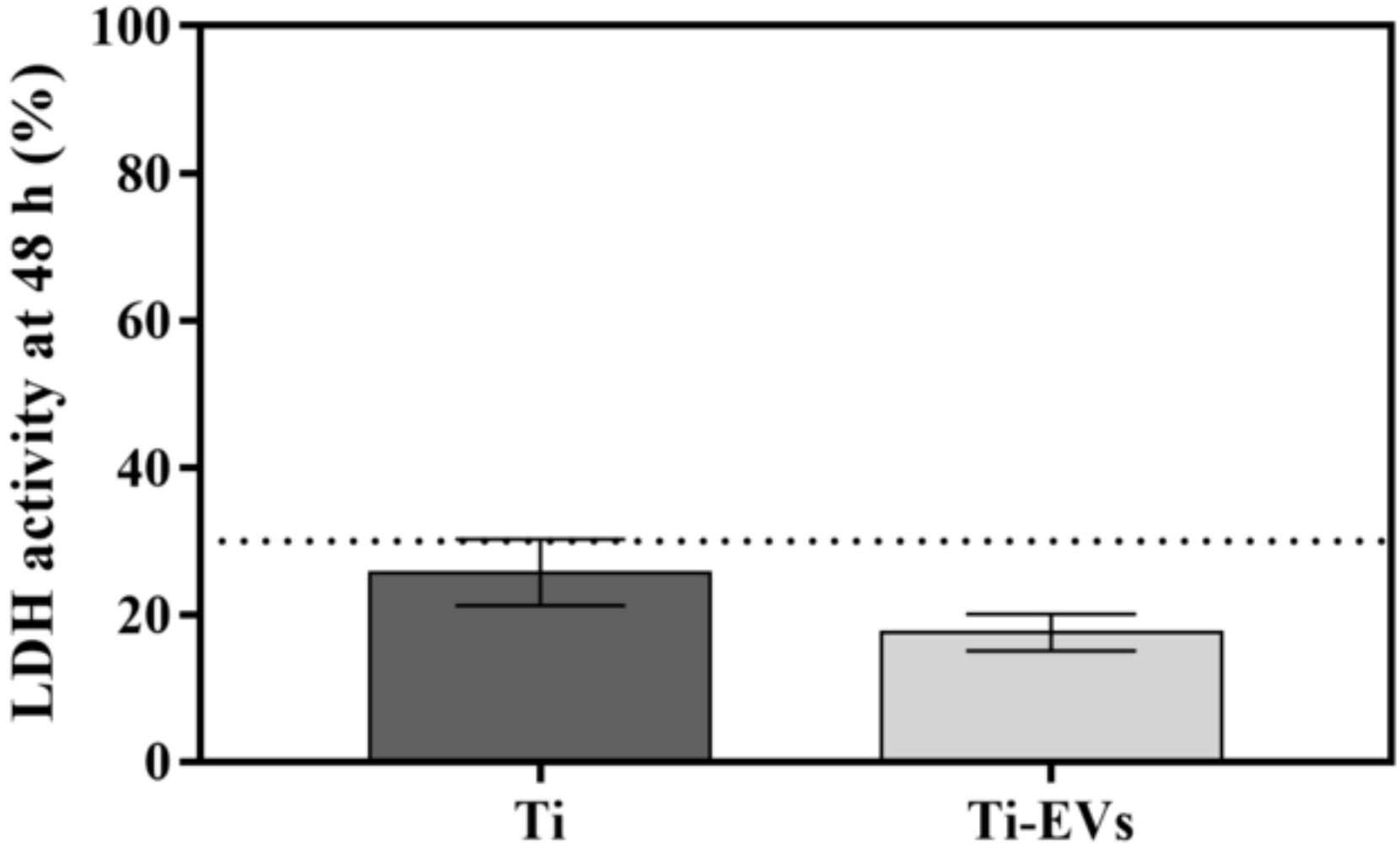


Figure 4



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Palma de Mallorca, 29th of June 2021

Dear Editor,

Please find enclosed a revised version of the manuscript entitled " **Platelet-derived extracellular vesicle functionalization of Ti implants**" by Miquel Antich-Rosselló, Maria Antònia Forteza-Genestra, Javier Calvo, Antoni Gayà, Marta Monjo and Joana Maria Ramis, Manuscript ID: **JoVE62781**, which we hereby submit to be considered for publication in *Journal of Visualized Experiments*.

In preparing this new version, we have taken into account all the editor and reviewer's suggestions. We also enclose an explanation of how the points have been dealt with.

We believe the manuscript has gained after taking into account the editor and reviewer's points. and we believe that the revised version of the manuscript will be of interest to the readers of *Journal of Visualized Experiments*.

Yours sincerely,

Joana M. Ramis

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Dear editor, we have revised the manuscript in order to correct any spelling or grammar mistakes.

2. Please provide an institutional email address for each author.

Thank you for the comment, authors' emails have been provided at the beginning of the main text.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

In agreement of the editor's advice, the text has been revised and all personal pronouns have been eliminated.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Nanodrop, etc.

Thank you for the advice, in agreement of the editor's suggestion the commercial language that was on the former version of the manuscript (Nanodrop and Sepharose) were eliminated from the main text.

5. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

Thank you for your recommendation, an ethics statement has been introduced before the protocol steps.

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes. For example, 1. EVs isolation from PL, 1.1. Large bodies removal followed by steps 1.1.1, 1.1.2, etc.

In agreement of the editor's suggestion, the numbering of the main text was adjusted.

7. Line 92-94: Please specify the methods for the characterization of fractions. Add references if necessary.

Thank you for your advice. Proper changes have been addressed in the main text. The characterization methods used are total protein quantification and immunodetection, thus this information has been referenced.

8. Line 106: Please specify the volume of the diluted EVs.

In agreement with the editor's suggestion the volume of the diluted EVs has been added to the mail text.

9. Line 125: Please elaborate on the washing step. How is it performed?

In the new version of the manuscript, washing steps have been further elaborated to clarify the protocol to the readers.

10. Line 150-151: Please specify the volume of HNO3 used. Is the volume used similarly as mentioned in the note (lines 126-127)?

In the new version of the manuscript a note has been added in order to clarify this step.

11. Line 160: Please specify what is meant by the "machined" side. Please specify if it is machined as a part of the protocol or available as such. If machining requires elaborate steps, consider adding a citation.

In agreement with the editor's advice, the term "machined" has been clarified in the main text (Section 3). Ti coins are commercially available with a machined side due to the production system, thus, this is not a part of the protocol.

12. Line 163: Please specify the vortex conditions (time, agitation, etc.).

Thank you for your recommendation. A short pulse was performed, changes have been addressed in the main text.

13. Line 180: Please specify the time points used in the study.

In agreement of the editor's advice, the different time points used in this study have been added in the main text.

14. Line 190: How are the cells trypsinized?

In the new version of the manuscript, the trypsinization step has been further developed in the main text to avoid confusion.

15. Line 195: How is the concentration of the cells determined?

Thank you for your question, detailed explanations have been added in the main text.

16. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

The suggested changes have been addressed on the main text.

17. Figure 1: Please revise the unit "xg" to "x g" leaving a single space between x and g (italics). Please include a space between "4°C". It must be "4 °C".

In agreement of the editor's suggestion, Figure 1 has been modified to introduce these changes.

18. Figure 2: Please remove the space between "30" and "%". Revise "2 hours" to "2 h".

In agreement of the editor's suggestion, Figure 2 has been modified to introduce these changes.

19. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

In agreement of the editor's advice, changes have been performed on the Table of Equipment and Materials.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This work reported by Miquel, Joana Maria Ramis et al. described a method about platelet-derived extracellular vesicle (EV) functionalization of Ti implants. Quite clear progress was reported in this work including EVs isolation, EVs characterization, titanium surface functionalization, and Ti surface characterization. It is a quite clear protocol, and I am in the opinion that this work could be accepted for the publication in Journal of Visualized Experiments. However, some further improvements are expected to clarify the protocol.

Major Concerns:

1. The process of 'Ti discs wash' was quite complex, and I suggest that the authors elaborate on the reasons for these actions. Whether the process has any effect on the surface of the material, such as the surface charge?

In agreement of the reviewer advice, the washing steps have been further detailed to clarify their performance. This is a generic protocol for implants washing that was adopted in our research group from the instructions received from an implant manufacturer under a research contract. The main goal of this step is to eliminate possible molecules adhered on Ti surface in order to ease EV interaction and physisorption onto the implant.

2. It is suggested that the author provide some methods to evaluate the effect of EVs drop casting Ti implants.

In agreement with the reviewer's suggestion, we have provided some methods to assess the effect of EVs drop casting Ti implants on the Discussion section of the new version of the manuscript. Suggested methods include the detection of osteogenic markers as prove of functionality.

3. I suggest that the author add some evidence to illustrate the morphology of EVs on the Ti implants after modified by the drop-casting method.

We agree with the reviewer's advice, usually, transmission electron microscopy (TEM) or atomic force microscopy (AFM) are the techniques used in order to visualize the morphology of EVs. However, these techniques are not directly compatible with Ti discs implants. On the one hand, TEM imaging requires thin samples that cannot be achieved from metallic samples in order to achieve good resolution of vesicle

structures in the nanoscale[1]. To section metallic biomaterials or samples that combine hard and soft components, a cutting/grinding-based histological sectioning is used, but the slides that can be obtained are of 10-15 µm, which is too thick for TEM analysis.

On the other hand, such images could be achieved by AFM, however, in our hands Ti rugosity hinders optimal resolution to allow EVs visualization. Nevertheless, we are currently optimizing our protocol and we hope to report proper EVs visualization on the implants in future articles.

4. There are differences in the shape of Ti implants and Ti discs, whether drop casting is still effective for the implants with different shapes?

We agree with the concerns of the reviewer. This study reports a valid methodology that supports a feasible binding of EVs to flat Ti surfaces. This methodology addresses a research lab method for a performance at small scale. However, scalability of this methodology to be performed in industrial facilities would probably change into a dip-coating approach. Although further experiments should be performed to assure a homogeneous surface binding using different shapes and rugosities. However, before raising concerns about their industrial translation, further functionality and efficacy results should be reported in small scale experiments.

5. '2.4 Seed 200 µL of cell solution onto the Ti implants' in 'Ti surface characterization'. Is the cell solution added to the Ti implant (titanium discs, c.p. grade IV, 6.2 mm diameter and 2 mm height)? Is it difficult to prevent cell suspension from spilling from Ti implants? Besides, can cells grow on titanium implants? Why did the authors do this experiment with 10,000 cells, rather than 5,000 cells as is often reported?

We agree with the reviewer's concerns. In fact, we have designed the Ti discs in order to perfectly fit the wells of the 96-well plates in a way that little surface other than titanium's is available for cell growth. Furthermore, 10 000 cells were seeded to assure a nearly confluent cell growth, while other studies with lower cell density usually address proliferation or migration assays.

[1] Sadamatsu, S., Tanaka, M., Higashida, K., & Matsumura, S. (2016). Transmission electron microscopy of bulk specimens over 10 µm in thickness. *Ultramicroscopy*, *162*, 10-16.

Reviewer #2:

Manuscript Summary:

A potentially useful paper, However, some major revision should be made.

Major Concerns:

1. There is not enough evidence to support the successful extraction of platelet-derived extracellular vesicle.

We agree with the reviewer's concern. EVs characterization has been performed through NTA and total protein concentration, which are necessary to perform a correct Ti functionalization. It is true that the international society of EVs recommend a further characterization when functional effects are reported, characterizations that we have performed in previous reports using the same EVs source[2].However, the aim of this article is to focus on the new methodology used for Ti surface functionalization with EVs, rather than the functional effects of them. A NOTE has been added to the main text to clarify the EVs characterization methodology.

2. More characterization should be included to show the successful functionalization of extracellular vesicle on Ti implants.

We agree with the reviewer's advice, we have provided some methods to assess successful EVs functionalization on the Discussion section of the new version of the manuscript.

[2] Antich-Rosselló, M., Forteza-Genestra, M.A., Calvo, J., Gayà, A., Monjo, M., Ramis, J.M. Platelet-derived extracellular vesicles promote osteoinduction of mesenchymal stromal cells. *Bone and Joint Research*. **9** (10), 667–674, doi: 10.1302/2046-3758.910.BJR-2020-0111.R2 (2020)