Biotech Method

Improving biomaterials imaging for nanotechnology: rapid methods for protein localization at ultrastructural level

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Abbreviations: TEM, transmission electron microscopy; **AFM**, atomic force microscopy; **TFM**, tunnelling force microscopy; **NSOM**, near-field scanning optical microscopy; **SPM**, scanning probe microscopy; **EM**, electron microscopy; **GFP**, green fluorescent protein; **MMP-2**, matrix metalloproteinase 2; **MMP-9**, matrix metalloproteinase 9; **IFN-γ**, interferon gamma; **SP**, Standard Protocol; **NSP1**, New Simplified Protocol 1; **NSP2**, New Simplified Protocol 2; **PB,** phosphate buffer; **SE**, secondary electron; **BSE**, energy selective back-scattered electron.

Abstract

The preparation of biological samples for electron microscopy is material- and time-consuming because it is often based on long protocols that also may produce artifacts. Protein labeling for transmission electron microscopy (TEM) is such an example, taking several days. However, for protein-based nanotechnology, high resolution imaging techniques are unique and crucial tools for studying the spatial distribution of these molecules, either alone or as components of biomaterials. In this paper, we tested 2 new short methods of immunolocalization for TEM, and compared them with a standard protocol in qualitative and quantitative approaches by using four protein-based nanoparticles. We reported a significant increase of labeling per area of nanoparticle in both new methodologies ($H=19.811$; $p<0.001$) with all the model antigens tested: GFP ($H=22.115$; $p<0.001$), MMP-2 ($H=19.579$; $p<0.001$), MMP-9 ($H=7.567$; $p<0.023$), and IFN-γ ($H=62.110$; $p<0.001$). We also found that the most suitable protocol for labeling depends on the nanoparticle's tendency to aggregate. Moreover, the shorter methods reduce artifacts, time (by 30 %), residues and reagents hindering, losing, or altering antigens, and obtaining a significant increase of protein localization (of about 200 %). Overall, this study makes a step forward in the development of optimized protocols for the nanoscale localization of peptides and proteins within new biomaterials.

Keywords: Protein-based nanomaterials TEM Immunolocalization New protocols

1. Introduction

Nanotechnology has rapidly emerged as a powerful set of platforms with limitless potential to create new and highly specific solutions for several fields. These include the development of innovative therapies and new generation of drugs aimed to solve current limitations of the pharmaceutical industry. In nanomedicine, the development of nanocarriers for drug delivery opens the path to generate more effective and personalized treatments for important human and animal diseases [1-3]. In contrast with conventional drugs, the new generation of nanoscale drugs show improved stability, extended circulation time, reduced toxicity, high therapeutic efficacy, and cell targeting [4]. Specifically, natural and engineered peptides and proteins are among the most suitable building blocks for nanoparticle generation due to their full biocompatibility and degradability at physiological conditions and versatility to be engineered and produced in single step by inexpensive biological processes [1]. Moreover, protein-based materials can be tuned by modifying both production conditions and genetic cell background [5-8], to generate a wide spectrum of protein nanoparticles with different composition, structure, size, and shape adapted to specific uses in animal and human medicine [9-12]. During the last years, peptide- and protein based biomaterials have emerged as convenient nano- and micro- carriers fully compatible with cells, tissues, and entire organisms [13]. They have great loading capacity and a high efficiency in differential transport, biodistribution, and/or release compounds with therapeutic interest [1;14-17].

In the context of this drug delivery-oriented research, high resolution imaging is crucial for resolving the spatio-temporal distribution, for quantifying the amount of peptide or proteins building blocks, or for understanding their form at nanoscale level, and for predicting and studying their potential function in different complex biological systems. The large number of high resolution imaging devices coupled with a wide variety of detectors allows obtaining 2D or 3D images of a wide spectrum of liquid and solid samples. Then, common nanoscale imaging has been used to characterize surface roughness, geometry (size and shape), and ultrastructural details of liquid and solid nanobiomaterials at more or less native state by atomic force microscopy (AFM), tunnelling force microscopy (TFM), or near-field scanning optical microscopy (NSOM). Other types of scanning probe

microscopy (SPM) are used to study surfaces [18], whereas a wide variety of techniques and devices are available for obtaining other information at high resolution imaging reaching until few nanometres resolution [19-21]. Electron microscopy (EM), apart from being used to study surfaces, is probably the most used set of imaging techniques to determine inner details of biomaterials [22]. In fact, EM methods are powerful techniques that allow the imaging of cells, organelles, macromolecules, and atoms until native state. At present, transmission electron microscopy (TEM) is probably one of the most used set of techniques to characterize nanobiomaterials, achieving a spatial resolution adequate to localize, quantify, and map specific peptides or proteins at nanoscale level. In this context, cryomethods are a group of techniques to process EM samples that substitute conventional chemical methods (fixatives, metal oxides, epoxy resins, etc) for physical methods (high pressure, low temperature), thus increasing resolution, decreasing artifacts, and also allowing the visualization of the ultrastructure at nearly native state and to localize molecules or elements at ultrastructural level that may be altered with conventional methods [23;24]. Post-embedding immuno EM (iEM), probably the most common set of cryomethods for TEM, has been explored using different electrodense markers such as ferritin or peroxidase. However, colloidal gold beads of a determinate size range (from 1 to about 40 nm) coupled to immunoglobulins or proteins such as protein A or G, are the most used secondary antibodies trackers to localize antigens at nanoscale [25-27]. Nevertheless, despite the progress in this field, there is still an important requirement related to the development of improved techniques or methods, to obtain high resolution imaging of different types of samples [23;25;28-32] in a fast and effective manner. Although some specific contributions have displayed a time-, material-saving and improved results [23;33-35]; the optimization of TEM methods is clearly not enough achieved, especially for biomaterials. In this context, pre- and post-embedding immunolabeling protocols for TEM are a key question to link biochemistry, molecular biology, structural and ultrastructural studies or other newer research techniques with the spatio-temporal localization of peptides and proteins in a quantitative manner at nanoscale [24;25;35-37]. Here, we have developed two new post-embedding iEM methods for peptide and protein biomaterials based on the most used conventional approaches for TEM immunolabeling, aimed to the reduction of

time, reagents, and steps of labeling, that would in turn reduce costs, residues, and artifacts and increase protein localization (then, method efficiency and efficacy) of less altered nanobiomaterials. For that, we have used protein-based nanoparticles, also known as protein aggregates, as biomaterials model to evaluate the potential of the above mentioned protocols.

2. Material and methods

2.1. *Protein design*

The green fluorescent protein (GFP) and three proteins of bovine (*Bos taurus* sp.) origin, the catalytic domain of matrix metalloproteinase 2 (MMP-2; from Tyr110 to Asp45 NM_174745) and 9 (MMP-9; from Phe107 to Pro449 NM_174744) and the mature form of the interferon gamma (IFN-γ; from Gln23 to Thr101 NM_173925) were used, as described in [38]. All proteins were produced as protein-based nanoparticles (aggregated nanoparticles) in *Lactococcus lactis* NZ9000 *clpP*$^{-}$*htrA*$^{-}$ (*clpP-htrA*; Em^{R}) [38-40] (kindly provided by INRA, Jouy-en-Josas, France; patent nº EP1141337B1) using pNZ8148 vector (MoBiTech).

2.2. *Nanoparticle production and purification*

The production of protein-based nanoparticles was induced by the addition of 12.5 ng/ml nisin (Sigma-Aldrich) in bacterial cultures growing in M17 broth plus 0.5 % glucose at 30 ºC without shaking for 3 h. Once produced, protein nanoparticles were purified under sterile conditions and all incubations were carried out under agitation, using the purification protocol described by [41], and by adding at the beginning a mechanical disruption step by French Press.

2.3. *Labeling and imaging*

To reduce time and artifacts, we shorted long steps or and simplified or eliminate the non-crucial steps for the antigenicity maintenance and protein localization. Schematic summary of the three methods is shown in **Figure 1** and detailed in **Figure S1**. Samples of protein-based nanoparticles by GFP, MMP-2, MMP-9, and IFN-γ were processed:

1- Standard Protocol (SP): fixation in 4 % (w/v) formaldehyde (TAAB) and 0.1 % (v/v) glutaraldehyde (Merck) in phosphate buffer (PB; Sigma-Aldrich) for 30 min and stored in 1 % (w/v) formaldehyde prepared in PB. Samples were rinsed in PB, placed during 1 h with 20 mM glycine (Sigma-Aldrich) to quench free aldehydes, rinsed in PB, cryoprotected in graded series of sucrose (2 h in 0.7, 2 h in 1.4 and 12 h in 2.3 M; Sigma-Aldrich) solutions prepared in PB, and cryofixed in liquid propane in an EM CPC (Leica Microsystems). Vitrified samples were immersed in methanol (Merck) containing 0.5 % uranyl acetate (Polysciences Inc.) for 72 h at -90 ºC, washed with methanol for 3 h at -45 ºC, embedded in Lowicryl HM20 resin (Polysciences Inc.) for 24 h at -45 ºC, and polymerized with UV rays for 48 h at -45 ºC followed by 48 h at 25 ºC in an EM AFS automatic freeze substitution system (Leica Microsystems).

2- New Simplified Protocol 1 (NSP1): Rapid cryoprotection with glycerol (10 % 1 h, and 30 % 0.5 h, Sigma-Aldrich) solutions in PB without fixation with aldehydes, cryofixation in liquid propane and rapid cryodehydration (24 h in 0.5 % uranyl acetate at -90 ºC), rapid embedding (4 h) and same polymerization than SP. We eliminated steps of fixation and reduced cryoprotection and cryodehydration times, resulting in a reduction of about 25 % of time in relation to SP.

3- New Simplified Protocol 2 (NSP2): Direct cryofixation in propane, rapid cryodehydration without uranyl acetate step, embedding and polymerization as NSP1. We eliminate fixation and cryoprotection steps and we reduced cryodehydration and embedding times, resulting in a reduction of about 40 % of time in relation to SP.

Ultrathin sections (70 nm) of selected areas of semithin sections (1 µm) of each sample were obtained with Leica ultracut UC6 microtome (Leica Microsystems), placed on carbon-coated gold grids (200 mesh) and labeled in parallel for the 4 antigens of interest [22;38;42]. Briefly, sections were blocked in 1 % (w/v) bovine serum albumin (BSA; Sigma-Aldrich) in phosphate buffered saline (BSA/PBS) containing 20 mM of glycine (Sigma-Aldrich), incubated with the respective primary polyclonal primary antibody anti-GFP (#ab6556, Abcam), anti-MMP-2 (#AV20016, Sigma-Aldrich), anti-MMP-9 (#50560-RP01, Sino Biological Inc.), or

anti-IFN-γ (#ab9657, Abcam) at working dilution 1:25, 1:5, 1:5 and 1:2 respectively, washed in BSA/PBS, incubated in protein A coupled to 10 nm-gold particles (BBI Solutions) at working dilution 1:50, and washed firstly in PBS and then in deionized (MilliQ) water. Grids were contrasted with conventional uranyl acetate (15 min) and lead citrate (1 min), and examined with a TEM Jeol JEM-1400 (Jeol Ltd.) operating at 80 kV and equipped with a CCD Gatan ES1000W Erlangshen camera.

To imaging fluorescence at ultrastructural resolution, ultrathin sections of GFP samples in the same gold grids were observed without contrasting in a STEM mode in a Field Emission Scanning Electron Microscope (FESEM) Zeiss Merlin (Zeiss) operating at 2 kV. Images of morphology were obtained with a secondary electron (SE) detector and images of fluorescence were obtained with a back-scattered electron (BSE) detector. GFP fluorescence measurements were obtained by calculating the mean gray value from out part of nanoparticles (background) to the internal using Image J software (NIH Image).

2.4. *Quantitative analyses*

For antibody labeling detection, 20 images at same magnification (15,000X), for each sample and method, were randomly collected from 10 ultrathin sections. Then, for each sample and method, 30 protein nanoparticles were selected for quantification. In each nanoparticle, gold particles were counted and area was determined by binarizing images. For fluorescence determination, mean gray value was measured. Quantitative values were obtained for each method of GFP; measuring 30 randomly distributed 20 nm squared point inside the nanoparticles, then subtracting the mean value of 5 measures of 20 nm squared point from the background. Both analyses were carried out with Image J software (NIH Image).

2.5. *Statistical analyses*

Quantitative data are expressed as mean ± standard error. Comparison of methods of all proteins and for each protein and of fluorescence quantifications methods of GFP was made with Kruskal-Wallis H tests. Pairwise comparisons of methods for each protein were made with Mann-Withney U tests. Significance was accepted at $p<0.05$ and Bonferroni correction was applied for sequential

comparisons. All statistical analyses were performed with SPSS v. 18 for Windows.

3. Results and Discussion

3.1. *Improving protocols of post-embedding immunolocalization*

Pre and post-embedding immunolocalization TEM techniques are a set of current experimental methods that allow the visualization of antigens and offer a high-throughput manner to obtain high resolution images; however, they are material- and time-consuming processes. Some routine iEM cryomethods are a perfect example of this negative aspect. They have very long protocols, with steps taking from hours to days. One of the most common labeling techniques for TEM is the conventional protocol detailed in material and methods (SP) **(Figure 2)**. It has been widely used to localize different peptides and proteins in a wide range of biological systems such as bacteria [43;44], fungi [42;45], plants [46;47], culture eukaryotic cells [22;48], or animals [49;50]. Short versions of these long protocols were described for a wide variety of biological samples [23;31;35;36]. On one hand, by shortening the protocol to drastically reduce the costs of specialized staff, expensive reagents and devices, and potential artifacts in samples due to delocalization, lost or alteration of antigens and other ultrastructural details of samples [24;37]. Moreover, reducing the handling time of technically complicated methods also reduces potential human errors and exposure to toxins. On the other hand, by preserving the native state of the samples it may also conserve antigenicity due to less altered peptides and proteins, getting a higher labeling in less artifacted nanomaterials that allows the obtaining of robust quantitative and qualitative data [24]. Recently, several biomaterials, nano- and micro-structures with drug delivery and antimicrobial interest have been characterized by localizing a wide variety of peptides or proteins [13;43;51;52]. However, no specific studies of these new materials have ameliorated imaging techniques for the localization of antigens at nanoscale level. In our knowledge, the present study is the first reference of improved TEM immunolabeling protocols in biomaterials and opens a way of improved high resolution imaging by focusing on protein based nanoparticles with therapeutic interest.

3.2. *Protein nanoparticles as biomaterial model*

In this study, we have implemented two new procedures of pre-embedding immunolocalization for TEM (**Figure 1**) by using complex protein nanoparticles as a biomaterial model. The principal aim of our work was to increase protein localization and to reduce the complexity of sample handling in the detection of protein in intracellular nanoparticles. From a qualitative point of view, there are no changes in the aspect and size of samples independently of the method used **(Figure 2)**. However, protein nanoparticles were more electrodense when using the newly developed methods **(Figure 2)** that may be related to more conserved protein and, therefore, available for labeling. The study of this hypothesis, quantifying GFP fluorescence in the three methods in non-labeled ultrathin sections **(Figure 3)** revealed a clear pattern of GFP increases in nanoparticles when methods are shortened. Although more specific studies are needed with different antigens, we consider that the increase of electrodensity may be related with more protein being present in nanoparticles in shorter methods. This could be due to some protein being released from the biomaterials after the long and abundant steps of conventional labeling method. Finally, an unexpected advantage was found in the new protocols: a general reduction of cell debris around protein-based nanoparticles. Whereas in standard protocol, the nanoparticles of the four proteins were surrounded by non-labeled cell debris from bacterial cells; these residues were either reduced or disappeared in the new protocols (**Figure 2**). These results may be related to the non-fixation of samples that conserve protein deposits but not in the same manner as the rest of cellular components from the cell debris.

3.3. *Quantitative analyses of labelling and fluorescence*

The main result obtained in the present study is a significant increase of specific labeling with the new protocols compared with the standard protocol in all samples studied ($H=19.811$, $p<0.001$). For each protein, significant differences were observed all showing an increase of labeling in the new methods: at $p<0.001$ level GFP ($H=22.115$), MMP-2 ($H=19.579$), and IFN-γ ($H=62.110$), and at level $p<0.023$ for MMP-9 ($H=7.567$). Pairwise comparisons also detected significant increases of labeling of new protocols in relation to standard protocol (**Figure 3C**). Although all the proteins produced in this study are able to be self-assembled

to form protein nanoparticles (aggregates) in bacterial cytoplasm, different proteins have different aggregation propensity. Specifically, it has previously been published that GFP and IFN-γ have lower tendency to aggregate (50.27 % and 3.07 %, respectively) than MMPs (MMP-2: 100 % and MMP-9: 99.27 %) in protein-based nanoparticles [38]. Along this line, two clearly different patterns were observed by type of protein. Proteins with lower tendency to aggregate (GFP and IFN-γ) had heaviest labeling in the shortest method (NSP2), meanwhile, proteins prone to aggregate (MMP-2 and MMP-9), are better detected in NSP1 (**Figures 2** and **3C**). The increase of labeling indicates a high amount of protein conserved in protein nanoparticles without alteration and with intact antigenicity to specific primary antibodies, as demonstrated by the increase of GFP fluorescence (**Figures 3A** and **3B**). As recently described, the supramolecular organization of those nanoparticles consists of a fully resistant core in which protein with native or quasi native-like structure is embedded in [38]. This native protein has been described as it could be naturally released from nanoparticles under incubation in water-based salt solutions [5]. Therefore, in the case of biomaterials with lower tendency to aggregate (as much time they are in solution during the method), some proteins were lost or altered during the long steps in liquid and, consequently, there were less labeling. For this reason, in IFN-γ and GFP nanoparticles we observe more protein in the NSP2 in comparison with NSP1, because we have minimized the number of aqueous-based solutions (fixation, rinse, cryoprotection), thus reducing significantly the time in which nanoparticles may be rinsed and protein lost in solution. Nevertheless, in robust nanoparticles, such as the MMPs in which less protein release is observed, it is important to maintain the nanoparticle core, where most of the protein is found, more protected [5;38]. For MMPs, the conventional method maintains a high percentage of protein available for antibody detection. However, this immunolocalization significantly increases with NSP1 and NSP2 methods, although at lesser extent than for less aggregation-prone proteins. In relative terms, when using the news methods, there was a tendency towards the increase of labeling of about 200 % in the four antigens tested, with a dramatic increase (near 2,000 %) in the case of IFN-γ, which are the protein nanoparticle with less propensity to be formed. Overall, these results indicate that short methods are

useful to label peptides and proteins at ultrastructural level, but experiments must be designed depending on the physico-chemical characteristics of the antigen as well as on the 2D or 3D presentations of such biomaterials. As a disadvantage, new methods without fixation may be limited to biomaterials without complex cell structure. As alternative, these protocols may be coupled to a first rapid step of cryofixation such as high pressure freezing. The reduction or elimination of the rest of steps may be, then, explored in virtually all types of samples as an interesting improvement of time and cost of TEM protocols.

3.4. *Advantages and disadvantages of new methods*

Although these appealing new methods present some artifacts related to ice crystal formation (in a few of fields), as described in **Figure S2**, more labeling and less impurities around protein nanoparticles are detected (**Figure 2**). The result may be explained because these common artifacts in cryomethods destroy the nanoarchitecture but not necessarily decrease the antigenicity in these suboptimal parts of the samples. In contrast, optimal cryofixation areas deal with the formation of vitreous or amorphous ice that cryoimmobilize sample components without morphometric or compositional alteration. Then, the newly proposed methods enhance the sensibility of the technique, increasing the protein maintained in the sample and/or increasing the percentage of unaltered protein and potentially exposed to antibodies for localization. Moreover, it is important to point out that no background was observed in any sample after step cut down. The implementation of these shorter protocols offers important advantages such as fewer steps, samples in a nearly native state, fewer devices used, manipulation and specialized staff, and the reduction of potential artifacts; in other words, a cost-effective research. Despite reducing protocols steps, we also obtained an increase of labeling with the new methods, demonstrating that is possible to adapt standard techniques to new challengers of imaging at nanoscale level.

The labeling increase is a clear signal of technological improvement and may have a direct application in a wide spectrum of peptide and protein samples. From an economical point of view, only therapeutic peptides and proteins are a multibillion-dollar market with a strong growing prevision [53].They play a major

role in the present and future perspectives of medicine with important contributions in the treatment of diseases such as cancer or as efficient antimicrobial agents. However, most nanotechnological products fail to become a commercially viable because of the lack of characterization, among other reasons [54]. So, rapid and cheap methods such as ours may be applied for the adequate characterization of complex molecules and their future commercialization. Although, in this study, we have evaluated the efficiency of the newly developed protocols with protein-based aggregates, the study of peptides and proteins in a wide plethora of presentations at nanoscale such as self-assembling nanoparticles [14;52;55-57], bioinspired proteosomes [58;59], dendrimers [60], micelles [61;62], scaffolds [63], nanocomposites [3], coiled-coils [64;65], and vault nanocapsules [66;67], among others [13;53;68], could be substantially improved with these two new approaches. Although more specific studies are necessary, the results presented here focused on the use of general methods as a first screening of samples but with the mind in specific protocols to obtain best possible localization of peptides and proteins in a wide variety of samples.

4. Concluding remarks

The improvement of imaging methods to localize protein monomers, alone or nanostructured in form of protein nanoparticles or aggregates, as well as combined with other organic or inorganic nanostructures, is crucial to understand their form at nanoscale level and their function in biological systems with different organization (from cell cultures to entire organisms). On the other hand, since EM techniques continue to be among the most used set of high resolution imaging due to the realistic and easily interpretable 2D and 3D images, high versatility of biological and material samples, and wide type of morphological and compositional information, the interest to improve high-resolution techniques is crucial to obtain the best for nanotechnology studies in general and nanobiomaterials in particular.

The intensification of labeling obtained at nanoscale resolution together with a time and reagents reductions allows to improve the efficacy to map peptides and proteins at high resolution in virtually all types of nano- and micro-biomaterial

presentations such as particles, scaffolds, dendrimers, vault capsules, and/or composites. With a general increase of labeling near 200 % and time reduction of method of 30 %, the methods presented explore a more efficient way of high resolution imaging of biotechnological and pharmaceutical relevant peptides and proteins for nanotechnology uses.

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Figure captions

Figure 1: Schematic summary of the three methods presented in this study, detailing each step. Box widths represent time of step, and an approximate time of each step and entire protocol is also reported.

Figure 2: Representative micrographs of TEM immunolocalization at three magnifications of the four protein antigens (GFP, MMP-2, MMP-9, IFN--γ) by using the three methods studied: Standard protocol (SP), New simplified protocol 1 (NSP1) and New simplified protocol 2 (NSP2). Each protein nanoparticle was labeled with a specific antibody. Scale bars: left inset 200 nm; right inset 100 nm; lower micrographs 500 nm.

Figure 3: Representative micrographs of (**A**) nanoparticles morphometry and GFP fluorescence in the three methods, by using back-scattered electron (BSE) detector to quantify fluorescence and using secondary electron (SE) detector as reference of morphological aspect (Scale bars: 100 nm), and quantifications of (**B**) fluorescence by antigen area and method and (**C**) labeling regarding the three methods and four antigens tested: Standard protocol (SP), New simplified protocol 1 (NSP1) and New simplified protocol 2 (NSP2). The standard error is represented by either black or grey lines at each sample. Level of significant differences between three methods and each productive strains are indicated by superscripts (*p<0.05, **p<0.01, ***p<0.001).

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