Effects of cell line proliferation on the aggregation and stability of a hyaluronic acid solution (HA)/PLGA microparticles dispersed in the culture system

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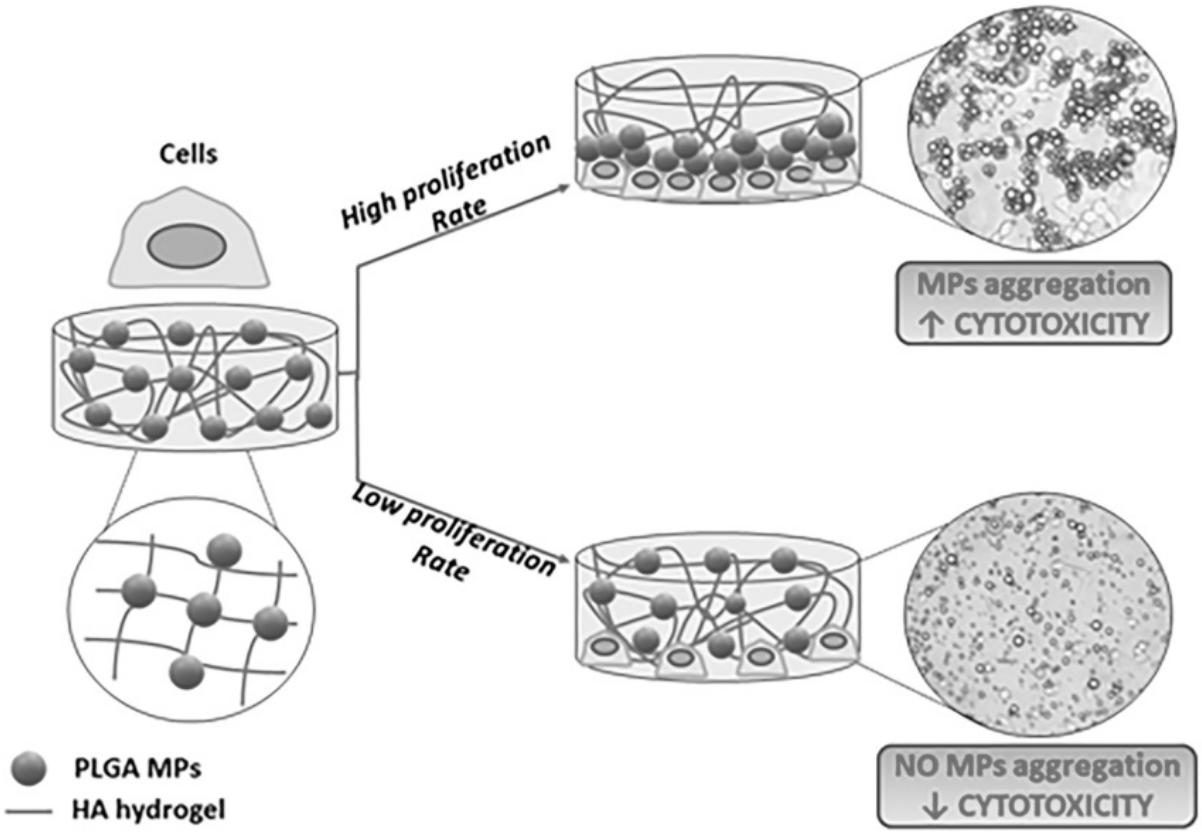
Abstract

Increasing efforts have been focused on development of novel therapeutic approaches based on a carrier mixed with polymeric microparticles (MPs) for promoting bioactive factors delivery. Polymers as poly (lactide-*co*-glycolide) are the most widely used due to their tunable physico-chemical properties and biomimetic character. Here, PLGA-MPs of different surface charges were dispersed in hyaluronic acid and their impact on the viability of four cell lines was studied. Our data reveal that the most evident effect is the influence of the cell line proliferation-rate on PLGA-MPs stability and aggregation, highlighting the crucial role of the biological context in affecting microparticles clustering and toxicity.



Keywords: Cell proliferation; cytotoxicity; hyaluronic acid; microparticle aggregation; PLGA microparticles

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1. Introduction

Micro-size particles of 1–200 μm range show great potential in several applications ranging from medicine and cosmetics to biotechnology. The aim is to produce materials and devices with new properties and functions by engineering their small structure[1]. In particular, the advantages of polymeric particles to deliver therapeutics and as diagnostic tools are evident, owing to their remarkable properties such as good biocompatibility, easy design and preparation, customizable structures and bio-mimetic character[2].

Moreover, polymeric particles are able to incorporate both small and large molecules, hydrophobic and hydrophilic drugs[3, 4]. This flexibility makes them particularly functional for the encapsulation of fragile drugs, such as nucleic acids and peptides, preventing their degradation and elimination from the body[5]. They can be also employed for targeting potent drugs, such as chemotherapeutic agents, providing their localization at the desired site of action and reducing the adverse effects of a systemic administration[6, 7]. Finally, polymeric particles have definitely revolutionized the field of controlled drug-delivery owing to their tunable

physico-chemical properties which provides controlled and sustained drug release, thus minimizing their fluctuation and frequency of administration and enhancing the compliance of patients[8].

Among all the biodegradable and biocompatible polymers, polyester is the most extensively investigated. Especially, Poly(lactide-*co*-glycolide) (PLGA) has been successfully used in form of microspheres in several clinically available products approved by the Food and Drug Administration (FDA), covering different therapeutic areas[9, 10].

PLGA is a copolymer between lactic and glycolic acids and has a negative charge at neutral pH[11]. Depending on the ratio of lactide to glycolide used for the polymerization, different forms of PLGA can be obtained; for example, PLGA 50:50 identifies the copolymer composed of 50% lactic acid and 50% glycolic acid. Among the different combinations, this copolymer exhibits the faster degradation rate (about 2 months)[12, 13] and its hydrolysis leads to two endogenous monomers (lactic acid and glycolic acid) which are easily metabolized via the Krebs cycle. Probably for this reason, the toxicity associated with PLGA MPs is expected to be minimal[14].

Another interesting property of PLGA MPs is their versatility, in fact they can be easily modified to improve their interaction with cell targets. The interaction between PLGA MPs and the cell membrane is a dynamic process regulated by nonspecific and weak bonds, such as electrostatic interactions and hydrogen bonding. It is known that modification of the MPs surface charge can improve their localization at the action site and then the interaction with specific cellular targets[8, 12, 15]. Despite the remarkable progresses in the field of PLGA MPs, the exploration of cellular response to MPs remains an attractive goal to optimize their therapeutic possibilities. In this context, the aim of the present study was to investigate the impact of the surface charge modification of PLGA MPs on cell behavior through*in vitro* experiments with different cell lines (NIH-3T3 fibroblasts, neuroblastoma N2A and pancreatic βtc3 and αtc1 cells). Given that PLGA MPs cluster when resuspended in cell media, we formulated a solution based on hyaluronic acid (HA), a polyanionic glycosaminoglycan ubiquitously expressed in human body and approved by the FDA as a gel for skin clinical treatments[16]. The dispersion of PLGA MPs in the HA solution avoid the aggregation phenomenon induced by the culture media, allowing us to investigate the cell-MPs interactions.

2. Experimental section

2.1. Materials

Poly(lactide-*co*-glycolide) (PLGA, Resomer® 50:50, ester terminated, M_W 38–54 kDa), Polyvinyl alcohol (PVA, Mowiol® 4-88, M_W ~31 kDa), Polydiallyldimethylammonium chloride (PDDA, 20 wt. % in H_2O), Polyvinylpyrrolidone (PVP, M_W ~40 kDa) were purchased by Sigma-Aldrich. Hyaluronic acid (HA, M_W 1.0×10^6) was purchased by IBSA. All solvents were of analytical grade, unless specified.

Four different types of cells were used: neuronal N2A (ATCC® CCL-131), fibroblast NIH-3T3 (ATCC® CRL-1658™), pancreatic αTC1 (ATCC® CRL-2350™) and βTC3 cells (kindly provided by Prof. Douglas Hanahan, Department of Biochemistry and Biophysics, University of California, San Francisco, CA, USA [17]).

2.2. PLGA microparticles (MPs) preparation

PLGA MPs were obtained by oil-in-water (*O*/*W*) emulsion method. First, the organic phase consisting of 40 mg PLGA in 0.8 mL ethyl acetate, was added dropwise to 1.6 mL of aqueous phase containing PVA (1 wt%) as stabilizer and 0.139 mL of ethyl acetate. The mixture was emulsified by Lab rotor-stator Homogenizer (IKA T-25 Ultra-Turrax Digital High-Speed Homogenizer System; 3000–25.000 rpm; power of 800 W), at 3400 rpm for 30 s. The obtained emulsion was added to 8 mL of a mixture containing 7.8 mL of water and 0.2 mL of ethyl acetate and magnetically stirred overnight to allow solvent evaporation. To remove any unconjugated PVA, MPs were centrifuged three times at 1000 rpm, for 10 min, at 4 °C. 2 µL of pellet corresponds to 0.5 mg of dried MPs.

2.3. Surface coating of PLGA MPs

PLGA MPs (20 µL of pellet or 5 mg of dried MPs) were suspended in 2 mL of 0.2 wt% PDDA in water or in 2 mL of 10 wt% PVP in water. The suspensions were stirred for 2 h at room temperature and purified twice by centrifugation (10,000 rpm for 5 min) at 4 °C.

2.4. Preparation of HA solution containing MPs

9 mg of HA were dissolved in 0.9 mL of water and kept under continuous stirring for 2 days, in order to obtain a homogeneous solution (final HA concentration 1 wt %). 3.6 µL of MPs pellet (0.9 mg of dried MPs) were added to the HA solution under mechanical stirring. The final concentration of MPs in the HA solution was 0.1 wt%.

2.5. MPS characterization and morphology evaluation

The morphology of PVA-, PDDA-, PVP-modified MPs alone or in combination with HA solution was analyzed by optical microscopy and their size and area measured by ImageJ software using the analyze particles plug-in. 2.5 µL of MPs were dispersed on a glass coverslip and optical micrographs were acquired

by inverted phase-contrast microscope (Leica microsystems DMIL LED) at 40× magnification with a digital Leica DFC 295 camera.

ζ-potential measurements were performed at 25 °C in low volume disposable cuvettes using a Malvern Zetasizer Nano ZS90 instrument, equipped with 633 nm solid state He–Ne laser at a scattering angle of 90°. Analyses were performed in water (viscosity: 0.8872 Cp, refractive index: 1.33). The charge measurements were averaged from at least three

repeated measurements and were performed after 2 and 24 h after dispersion in water.

2.6. Cell culture conditions

Neuronal N2A cells were cultured in DMEM high glucose, supplemented with 10% fetal bovine serum, 1% glutamine and 1% penicillin–streptomycin. Fibroblast-like NIH-3T3 cells were grown in DMEM low glucose, 10% fetal bovine serum, 1% glutamine and 1% penicillin-streptomycin. Pancreatic βtc3 cells were incubated in RPMI 1640 medium, 10% heat inactivated fetal bovine serum, 1% glutamine and 1% penicillin–streptomycin. Pancreatic αtc1 cells were cultured in DMEM high glucose, 10% heat inactivated fetal bovine serum, 1% glutamine and 1% penicillin–streptomycin. Before plating, cells were counted in order to obtain a density of 1.0×10^7/mL cells for βtc3, NIH-3T3 and αtc1 and 5.0×10^6/mL for N2A. Cell suspensions were mixed 1:1 with the HA solution alone or containing the different PLGA MPs (final concentration 0.5 wt% HA and 0.05 wt% MPs). Samples were seeded in 96-well plates (100 µL per well) and incubated in a humidified atmosphere containing 5% of CO_2 at 37 °C.

2.7. Evaluation of MPs aggregation rate

The aggregation rate was evaluated after 2, 24 and 48 h of culture, by optical microscopy (Leica DMI 4000B). Images were taken using 20× objective and collected with a Leica LasX software.

2.8. Cell viability assay

Cell viability was assessed by MTT test, at 24 and 48 h after plating. MTT (3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added in each well (final concentration of 0.5 mg/mL) and cells were maintained at 37 °C for 4 h in a humidified atmosphere containing 5% CO_2. Formazan crystals were then dissolved by overnight incubation with a solubilizing solution (10% SDS, 0.01 N HCl) and absorbance measured at 540 nm using the plate-reader TECAN infinite® F500. Experiments were performed in triplicate and data are expressed as fold increase over control sample (HA).

2.9. Statistical analysis

Data are presented as means ± SE of at least three independent experiments. Statistical comparisons were performed using analysis of variance, followed by multiple post hoc comparison analyses carried out using

Tukey's test (GraphPad Prism). The difference was considered statistically significant when the p value was < 0.05.

3. Results and discussion

3.1. Generation of PLGA MPs and inclusion in HA solution

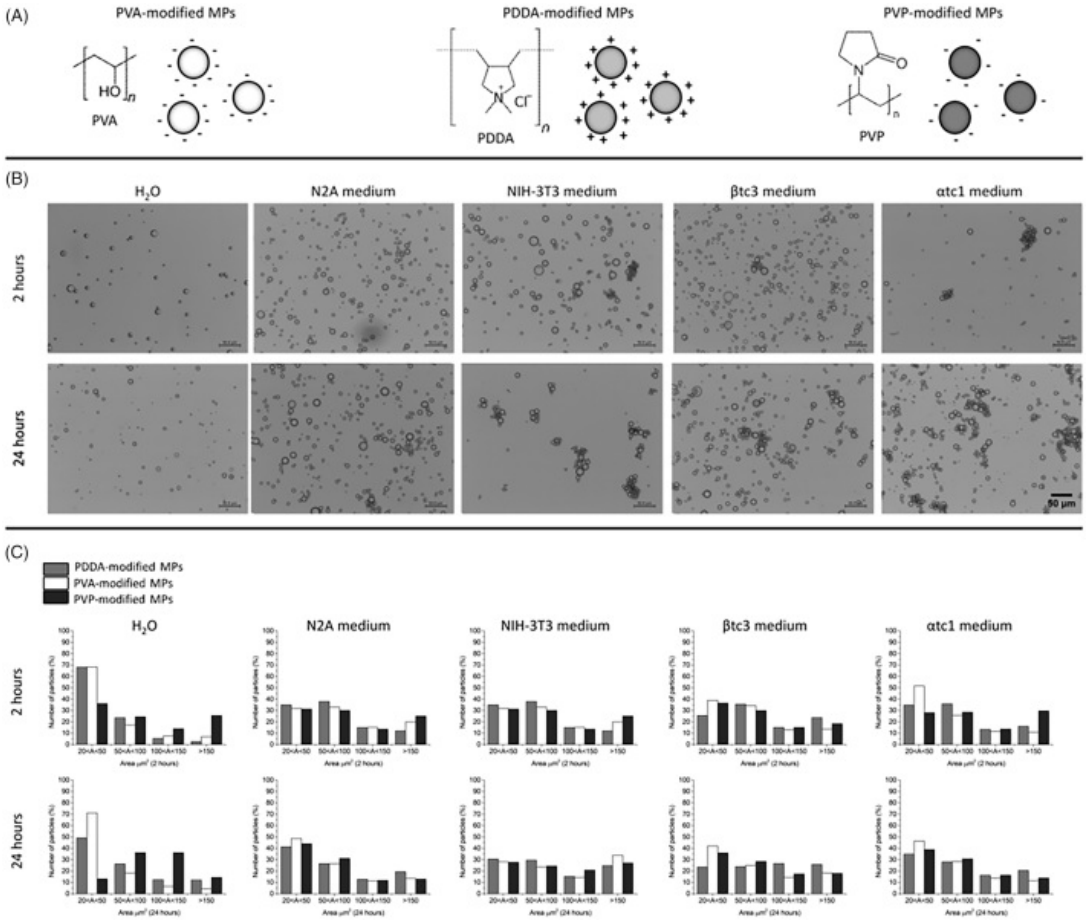
PLGA MPs were prepared by emulsification-solvent evaporation method, as described in Materials and Methods section. The polymer was dissolved in ethyl acetate and the oil-in-water emulsion (O/W) was prepared by adding water containing a stabilizer (PVA) to the polymer solution. The microsized droplets were obtained by homogenization, the solvent was evaporated, and the MPs collected by centrifugation. The microsized dimension allowed us to easily study the modification of PLGA MPs by optical microscopy, after their dispersion in water or in cellular media. Morphological analysis confirmed the presence of spherical particles with and average diameter of 5 µm and a net negative ζ-potential (Table 1).

Table 1. Diameter and ζ-potential of synthesized MPs, after 2 and 24 h from the dispersion in water.

	Diameter (µm)		ζ-potential (mV)	
	$t = 2$h	$t = 24$h	$t = 2$h	$t = 24$h
PVA-modified MPs	5.0 ± 2.2	4.2 ± 2.2	–20.5 ± 2.9	–18.8 ± 1.2
PDDA-modified MPs	3.2 ± 1.5	5.0 ± 2.7	10.8 ± 3.4	10.3 ± 1.9
PVP-modified MPs	4.5 ± 2.5	9.3 ± 3.2	3.1 ± 2.2/–10.8 ± 2.4	–17.5 ± 1.6

In order to obtain MPs with different surface charge, the PLGA MPs were coated with other polymers (Figure 1A). PDDA was used in order to generate a net positive charge. It is a homopolymer of diallyldimethylammonium chloride with a positive charge density due to the presence of quaternary amine and interacts with negative charges of PLGA through nonspecific electrostatic interactions. The ζ-potential of the final system is positive, as shown in Table 1. We also modified the PLGA MPs surface with PVP, a water-soluble polymer of *N*-vinylpyrrolidone. Depending on the amount of PVP absorbed on PLGA, the ζ-potential of this system ranges between −10.8 and +3.1 mV (Table 1). However, after 24 h, the ζ-potential returns to the original PLGA MPs value, conceivably due to the progressive PVP shell degradation.

Figure 1. (A) Schematic representation of synthesized MPs. (B) Representative optical micrographs of PVA-modified MPs after 2 and 24 h from dispersion in water and in the reported media. Bar: 50 µm. (C) Percentage frequency distribution by area (µm^2) of PDDA- (light grey), PVA- (white) and PVP (dark grey)-modified MPs after 2 and 24 h from dispersion in water or cell media. The distribution is normalized to the total number of particles counted in each specific preparation at the considered time point.



The stability of PVA-, PDDA- and PVP-modified MPs in water and in cell media was analyzed at 2 and 24 h. Since cell experiments were performed with N2A, NIH-3T3, βtc3 and αtc1 cells, PLGA MPs stability was studied in their respective media. Figure 1B shows the optical images of PVA-modified MPs and Figure 1C reports the frequency distribution of the particles' areas at the two time points in water and in the culture media. It is important to underline that the reported distributions are relative to the total number of particles counted at the considered time point, in each specific preparation. The analysis revealed that after 2 h, about 70% of PVA- and PDDA-modified MPs had an area range of 20–50 µm^2 in water, while only about 35% of PVP-modified MPs presented the same area range. After 24 h, the area range of PVA-

modified MPs remained unchanged, while it became larger in PDDA- and PVP-modified MPs (Table 1; Figure 1C). In general, the area of PVP-modified MPs was considerably greater than the

PVA- and PDDA-modified MPs areas since the uncharged PVP absorbed on PLGA MPs surface forms an outer shell through weak interaction in a dynamic equilibrium with the environment. Because of the high affinity of PVP for water, the PVP-modified MPs area increases but the surface charge becomes more negative due to the progressive PVP shell degradation. Conversely, the PDDA-modified MPs interaction is stronger because of electrostatic interaction, therefore, the outer PDDA shell is stable, and the surface retains the positive charge, even after 24 h (Table 1).

MPs were generally larger in biological media than in water, since only 40% of MPs had an area ~~size~~ range of 20–50 μm^2 after 2 and 24 h, regardless of the surface coating. Optical images revealed that MPs formed aggregates after 24 h in cell media, thus suggesting that the increased MPs ~~size~~ area measured in this condition was probably due to the MPs self-aggregation (Figure 1B and Figure S1). The phenomenon was particularly evident in the NIH-3T3 and αtc1 culture media and the maximum aggregation trend was observed with PVA-modified MPs. Among MPs, the PVA-modified MPs form the largest aggregates (Figure 1B), while the PDDA-modified MPs the smallest (Figure S1a).

A tentative explanation for the MPs aggregation observed in biological media during time is here hypothesized. The MPs dispersion in a medium favors the adsorption of proteins on the MPs surface. This outmost layer screens the surface charge and inhibits the electrostatic repulsion among the MPs, promoting, via macromolecule bridging, the MPs aggregation[18]. In agreement with this possibility, when we measured the ζ-potential of the samples (Table 2), ~~that~~ which is particles maintained in media, we found that all of them had a negative ζ-potential very similar to that of their respective media. Interestingly, the presence of PDDA-modified MP, which bear positive charges, causes a small modification of the system ζ-potential, probably because the electrostatic repulsion is low, and the system reaches the equilibrium more easily. For this reason, the smallest aggregates were obtained with PDDA-modified MPs. On the other

hand, PVA- and PVP-modified PLGA MPs, with their negative charges, probably destabilize the equilibrium of the system which reacts by shielding their surface charge and, consequently, the electrostatic repulsion.

Table 2. ζ-potential of synthesized MPs in different culture media.

	ζ-potential (mV)			
	N2A medium ζ= −4.7 ± 1.1	NIH-3T3 medium ζ= −6.3 ± 0.79	βtc3 medium ζ= −7.6 ± 0.26	αtc1 medium ζ= −8.8 ± 1.9
PVA-modified MPs	–4.3 ± 0.52	–3.4 ± 0.19	–4.2 ± 0.24	–3.5 ± 0.26

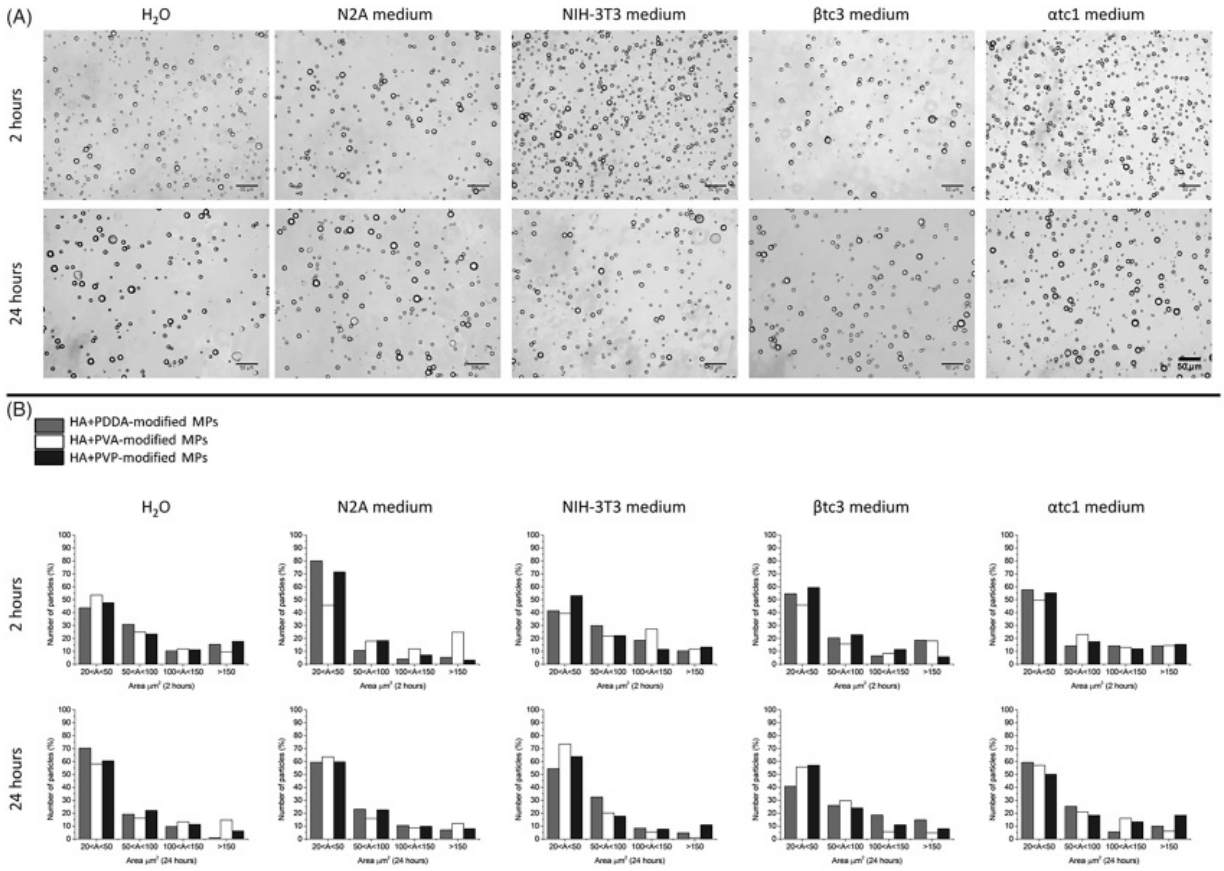
	ζ-potential (mV)			
	N2A medium ζ= −4.7 ± 1.1	NIH-3T3 medium ζ= −6.3 ± 0.79	βtc3 medium ζ= −7.6 ± 0.26	αtc1 medium ζ= −8.8 ± 1.9
PDDA-modified MPs	–5.7 ± 0.61	–6.2 ± 0.055	–7.2 ± 0.64	–6.3 ± 0.22
PVP-modified MPs	–3.3 ± 0.49	–3.4 ± 0.26	–4.3 ± 0.19	–3.5 ± 0.26

This phenomenon can be considered an example of “bottom up” self-assembly: the particles arrange themselves into bigger structures by exploiting physical or chemical interactions between the units to find the lowest-energy configuration[19]. This process is mediated by non-covalent interactions, including van der Waals and electrostatic interactions, that involve both the assembling units and the surrounding solvent molecules. Although these interactions are individually weak, their multiple cooperative actions trigger the formation of stable assemblies[20, 21]. In our system, the surrounding environment may significantly influence the outcome of the biomolecular self-assembly, in fact, in NIH-3T3 and αtc1 culture media, where the aggregation of PVA- and PVP-modified MPs was more evident, the ζ-potential reached values around –3.5 mV indicating increased macromolecule bridging.

In order to formulate a product without MPs aggregation, we included the PLGA MPs in a HA-water solution. Since the HA solution is apparently viscous, it forms a stable microenvironment around the particles, thus reducing the interactions between particles and biological medium and limiting the self-aggregation.

The percentage frequency of MPs with area between 20 and 50 μm^2 was similar for the different MPs generated, both in water and in cell culture media (around 60–70%), and no aggregation phenomena were observed in the HA formulations (Figure 2; Figure S2). Therefore, when MPs are embedded in the HA solution, the media composition does not influence the MPs behavior.

Figure 2. (A) Representative optical micrographs of HA + PVA-modified MPs after 2 and 24 h from dispersion in water or in the reported media. Bar: 50 µm. (B) Percentage frequency distribution by area (µm^2) of PDDA– (light grey), PVA– (white) and PVP (dark grey)-modified MPs in HA after 2 and 24 h from dispersion in water or cell media. The distribution is normalized to the total number of particles counted in each specific preparation at the considered time point.



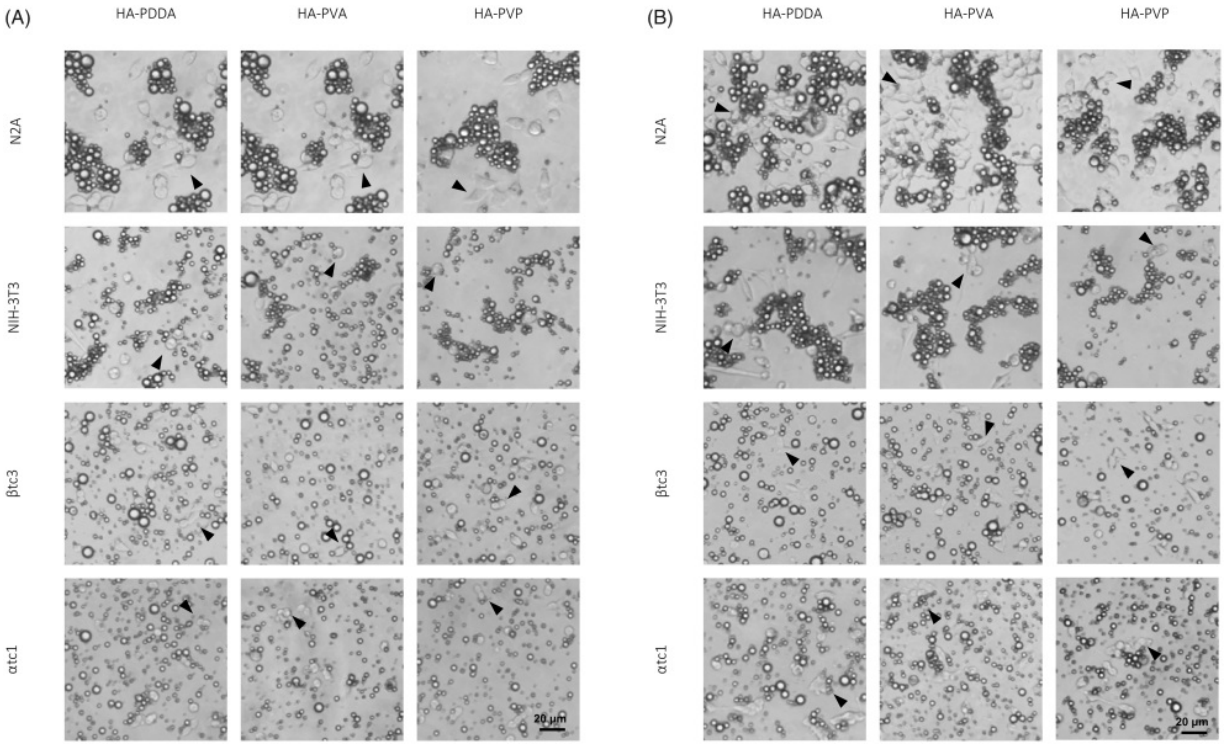
3.2. Cytocompatibility studies of PLGA MPs in HA solution

We investigated the behavior of PVA-, PDDA- and PVP- modified MPs dispersed within the HA solution in the presence of cells. Four cell lines, distinct for embryologic origin and proliferation rate, were selected and tested: neuronal Neuro2A, fibroblast NIH-3T3 and pancreatic αtc1 and βtc3 cells. Neuro2A (herein N2A) cells derive from the mouse neuronal crest and are extensively employed for studying neuronal differentiation and function. The high proliferation rate makes them a useful model for cytotoxicity studies[22]. NIH-3T3 are fast-growing mouse embryonic fibroblast cells widely used in material/cell interaction research[23]. βtc3 and αtc1 are both mouse pancreatic cell lines characterized by a low proliferation rate and useful for long-term cultures. Cell suspension was mixed 1:1 with the HA solution containing the different PLGA MPs and the MPs behavior as well as the cell–MPs interactions were evaluated by microscopy at 2,

24 and 48 h after plating.

As shown in Figure S3, no significant aggregation of MPs was detected during the first hours of culture. However, 24 and 48 h after plating, MPs self-aggregation became evident in N2A and NIH-3T3 samples, although with differences in these two cell lines (Figure 3). Indeed, in the presence of N2A cells, the MPs formed large complexes as early as 24 h after seeding, regardless of the presence of HA solution, the particle surface modification and the time in culture. Instead, the MPs self-aggregation was affected by the MPs coating and the time in culture when NIH-3T3 were employed.~~Indeed~~In particular, large MPs aggregates were already detectable at 24 h with PDDA-modified MPs, after 48 h with PVA-modified MPs, and seldom formed with PVP-modified MPs. As shown inFigure 3, no aggregation phenomena were observed in βtc3 and αtc1 cells, regardless of the MPs coating and the time in culture.

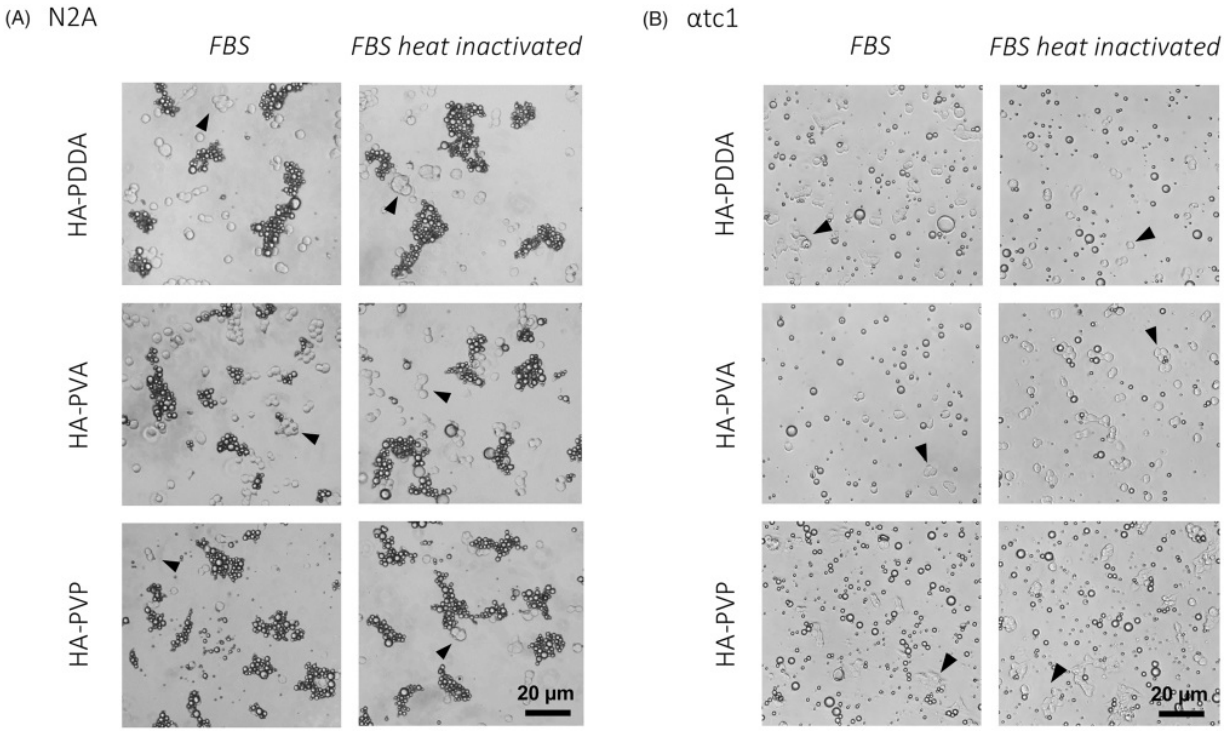
Figure 3. Representative light microscopy images of the HA solution loaded with PDDA–, PVA– and PVP-modified MPs mixed with N2A, NIH-3T3, βtc3 and αtc1 cells, 24 (A) and 48 (B) h after plating. Arrowheads indicate cells within the HA/MPs solution. Bar: 20 µm.



Since cells were cultured in four different media, we first excluded that aggregates formation was due to differences in media composition. The most important and discussed component of culture media is the fetal bovine serum (FBS); it provides water soluble and insoluble nutrients, inorganic salts, growth factors and hormones promoting cells growth but also contains proteases that may impact MPs stability. Since it is isolated from animal donors, its composition deeply depends on the diet and the animal environmental

conditions[24, 25]. Therefore, it is possible that batches-to-batches variability or heat-inactivation (1-h treatment at 56 °C) might influence not only the cellular function, but also the MPs stability and aggregation. We tested this possibility in N2A and αtc1 cells, as they grow in the same culture medium but the FBS is heat-inactivated only in αtc1 one. Optical images revealed that the MPs self-aggregation occurred only in N2A cells (not in αtc1), regardless of FBS batches or its inactivation (Figure 4).

Figure 4. Representative optical images of the HA solution loaded with PDDA-, PVA- and PVP-modified MPs mixed with N2A and αtc1 cells, 48 h after plating. Bar: 20 µm. (A) N2A cultured in FBS (left panel) and FBS heat inactivated (right panel) supplemented media. (B) αtc1 cultured in FBS (left panel) and FBS heat inactivated (right panel) supplemented media. Arrowheads indicate cells within the HA/MPs solution.



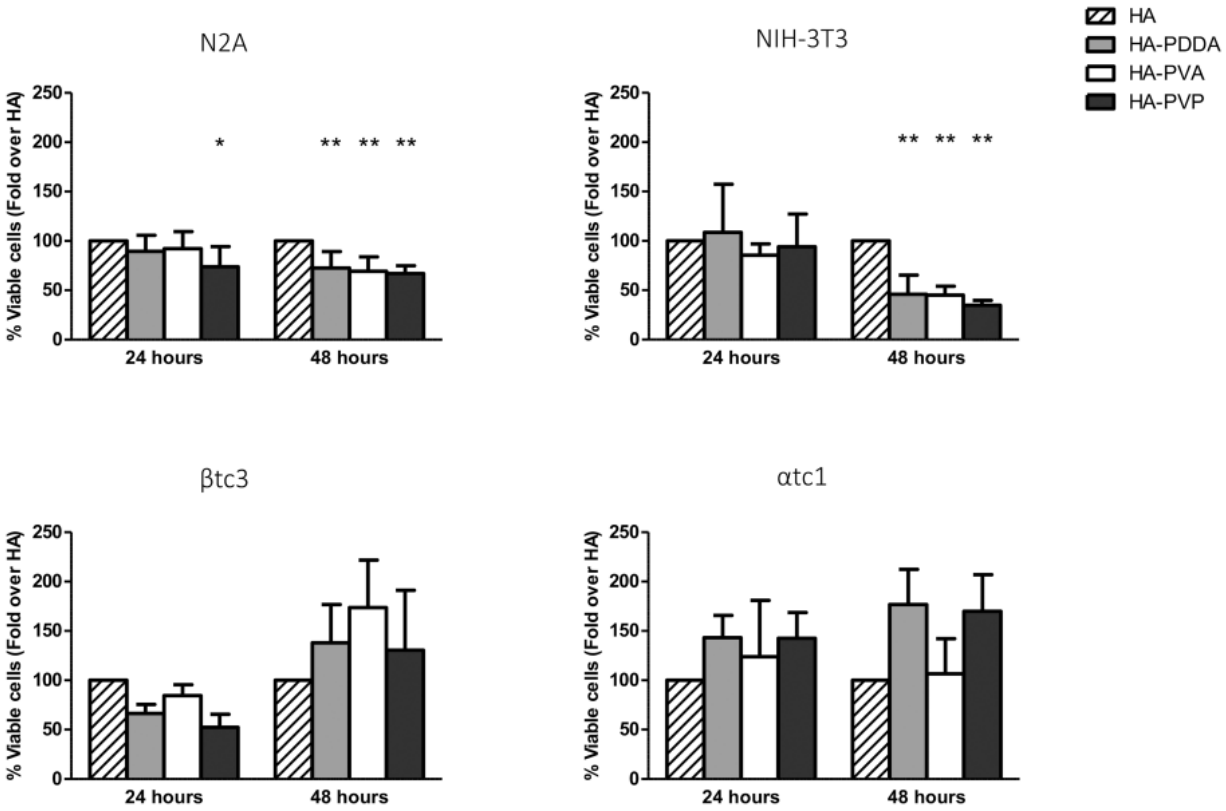
As the proliferation rate of N2A and NIH-3T3 cells (where aggregation was observed) is higher than that of pancreatic αtc1 and βtc3 cells, we hypothesized that MPs aggregation can be affected by the cell density. To test this possibility, N2A and βtc3 were plated at two different densities ($5.0\times10^6/1.0\times10^7$ per mL for N2A; $1.0\times10^7/1.5\times10^7$ per mL for βtc3) and MPs aggregation monitored

during time in culture. We found that MPs formed aggregates only in N2A cells, at both cell densities, but seldom formed in βtc3 cells, regardless of the surface coating and the time in culture (Figure S4).

Taken together, these data reveal that the MPs aggregation in presence of cells, does not rely on the surface particle chemistry, cell density or culture medium composition but it is strictly cell-type dependent. It is known that cells secrete a variety of cell-specific factors and enzymes that can actively modify the extracellular milieu, in particular the HA, thus promoting MPs clustering in a cell-specific manner. Interestingly, the proliferation rate of the examined cell lines is N2A > NIH-3T3 □ αtc1, βtc3 and correlates with the amount of aggregates formation; thus, in our system, the higher the proliferation rate, the greater and faster the MPs self-aggregation. Rapidly dividing cells often rely on a peculiar metabolism known as "aerobic glycolysis" to ensure energy for homeostasis and biosynthetic building blocks for daughter cells[26, 27]. It is characterized by an increased rate of glucose uptake and its preferential fermentation to lactate, which is secreted in the medium thus modifying the pH. Therefore, a local acidification or more generally the accumulation of specific metabolites during the time in culture may create a cell-specific microenvironment, which promotes MPs aggregation.

We finally investigated the effects of PDDA-, PVA- and PVP-modified MPs on cell viability by MTT test, at 24 and 48 h after plating; HA solution alone was used as control. Concerning N2A cells, only PVP-modified MPs significantly reduced cell viability after 24 h in culture (Figure 5), but all particles exerted a cytotoxic effect after 48 h (35±4% reduction of cell viability compared to HA alone; $p < 0.01$). A similar trend was observed in NIH-3T3 samples, as cellular viability measured at 48 h after plating was significantly lower in cells cultured in the presence of all the modified MPs than in control samples (50±6% reduction compared to HA; $p < 0.051$), regardless of MPs surface charges. Interestingly, in those cells where MPs did not form large aggregates, namely βtc3 and αtc1 cells, cell viability was comparable to control samples, thus suggesting that MPs cytotoxicity was probably associated with MPs aggregation rather than surface coating.

Figure 5. Quantification of cellular viability by MTT test after 24 and 48 h in culture. Cells were mixed with HA solution without or with MPs (HA, HA + PDDA-, PVA- and PVP-modified MPs). Data (mean values ± SE) are expressed as fold change over control (100% viable cells) ($n = 3$, in triplicate) (*$p < 0.05$, **$p < 0.01$ vs. HA).



The causal relationship between MPs aggregates formation and cytotoxicity is still under investigation.

A possibility is that MPs, by clustering around cells, can trigger cell death ~~trough reduction of~~by decreasing oxygen and nutrients availability, but it may also be possible that self-aggregation is simply the consequence of cell death. Indeed, loss of membrane integrity is the hallmark of cell death and causes the release in the extracellular environment of macromolecules such as DNA fragments but also enzymes and metabolites which in turn may modify the microenvironment and/or the MPs, thus promoting their aggregation during the time in culture.

4. Conclusion

In summary, the surface charge of MPs affects the particles interaction with the surrounding environment, their stability and self-assembly in large aggregates, particularly in complex media such as those of cells. However, once MPs are dispersed in HA, the cell type they interact with becomes the main factor

governing their stability and their self-aggregation. These results reveal that the MPs behavior is strictly related to the biological microenvironments where they reside, indicating that it may be extremely challenging to predict their behavior within the body.

We also suggest that the proliferation rate, rather than the cellular density by itself, is the key factor in triggering the formation of MPs aggregates during the time in culture, and this in turn induces cell death. Even though the mechanisms behind this phenomenon need to be further investigated, we provide a better understanding of the interactions between cells and MPs which may be useful for the development of new drug delivery systems. Particularly, the close relationship between cell proliferation, MPs aggregation and their cytotoxic effect, may be at the basis of new release treatments particularly relevant for cell therapy.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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