**Immune Profiling of Polysaccharide Submicron Vesicles**

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**ABSTRACT**

Alginate-(ALG) and chitosan-(CS) have been extensively used for biomedical applications; however, data relative to immune responses exerted by them are scarce. We synthesized a submicron vesicle system (SV) displaying a CS shell over an ALG core. Intravenous injection of these promising carriers could be a possible route of delivery; therefore, we evaluated their impact on human peripheral blood mononuclear cells (PBMCs). By this ex vivo approach, we established how SV chemical-physical characteristics affected the immune cells in terms of cellular uptake, viability and state of activation. By flow cytometry, we demonstrated that SVs were internalized by PBMCs with differential trends. No substantial necrotic and apoptotic signals were recorded and SVs weakly affected activation status of PBMCs (concerning the markers CD69, CD25, CD80 and the cytokines TNF-αand IL-6), showing high immune biocompatibility and low immunomodulating properties. Our findings gain particular value towards the biomedical applications of SVs, and make these polymer-based structures more attractive for translation into clinical uses.

**KEYWORDS**

Alginate, chitosan, polysaccharides, synthetic vesicles, immune response, flow cytometry.

**INTRODUCTION**

In the past decade, nanotechnology has put its roots in the drug and medical device industry, pursuing nanomedicine as the use of nanoscale or nanostructured materials that, according to their structure, have distinctive medical applications 1,2. In this scenario, nanotechnology methods have resulted in nanoscale materials, both of biologic derivation or synthetically created, with new, desirable and exploitable properties, suitable for a multiplicity of therapeutic 3-5 and diagnostic approaches 6-7. However, their potential immunotoxicity has not received sufficient consideration 8,9. Thus, the study of undesirable interference between man-made nano objects and biological nano-scale and micro-scale structures is a critical point 10. As it is well-known, the cells of the immune system (IS) recognize foreign substances and initiate the activation of innate immune response, i.e. the inflammation mediated by cytokines, chemokines and lipid mediators 11. Remarkably, nano-materials (NMs) can interact with various components of the IS and either enhance or inhibit its function 12-14. Composition, shape, size and mainly physico-chemical properties of the NMs play critical roles in influencing the IS response 15-18. The NM biological impact can also be affected by impurities, generated as by-products in NP synthesis 19, or by endotoxins 20. The modulation of immune function by NMs can be useful or detrimental, depending on the intended use 12. NMs can be designed to act as immunomodulators to serve specific functions (e.g. vaccine adjuvants, anti-inflammatory or immunosuppressive drugs). However, NMs engineered to act as cell or drug delivery systems, and not intended to interact with the IS, may also alter its function. Multivalency, molecular weight, and molecular architecture control have been demonstrated to impact the biological interaction of, among other molecules, carbohydrate-bearing chains with their binding partners. This viewpoint underlines potential applications of carbohydrate-based polymers for immune modulation 21,22. On the other hand, NMs prepared from polysaccharides have been extensively exploited for drug delivery, because of their natural origin, chemical functionality, biodegradability, good biocompatibility and low- cost processes 23. Polysaccharides are naturally occurring carbohydrate-based polymers, repeated units joined together by glycosidic bonds. Common types are represented by algal (i.e. alginate, ALG), plant (cellulose, starch) and animal polysaccharides (chitosan, CS). They exhibit quite variable structures and properties, different reactive groups, a wide range of molecular weights and variable chemical composition 24. In addition, CS is characterized by mucoadhesive properties, resulting in a reversible structural reorganization in the protein-associated tight junctions 25. The delivery of anticancer agents, proteins, growth factors, antibiotics, anti-inflammatory drugs, as well as vaccine delivery and gene therapy, have been successfully achieved by CS-based carriers approach 26,27. Since these systems are often exposed to blood, they have to satisfy criteria of bio/hemocompatibility 23. As underlined by a number of authors, no standard immunotoxicity assays, specific to their nano size, has been described for NMs 28,29. Even so, the same studies routinely used to assess immunotoxicity of chemicals, medical devices and drugs can be applied to engineered NMs 14. In this context, we investigated sub-micron synthetic vesicles (SVs) displaying a CS shell over an ALG core in the form of polyelectrolyte complex (PEC). The production of microcapsules having a complex coacervate ALG–CS membrane and an ALG core differs with respect to the applied materials and processes 30. SVs were previously obtained by a microfluidic aided two-step method 31,32 and their effective drug carrier functionality was assessed on an acute kidney injury in vitro model 32. It is already known that, when CS represents the main/external component of the NM, the system holds a cationic surface, which considerably reduces its circulation time and bioavailability upon exposure to a biological fluid 23,33. Nonetheless, when anionic polysaccharides, such as hyaluronic acid (HA) or ALG are allowed to interact with CS, reduced protein adsorption and reduced rate of macrophage uptake can be recorded 23,34,35. Overall, NM surface interacting with blood, plasma or interstitial fluid proteins, may lead to conformational changes, the exposure of new epitopes, altered functions and/or avidity effects 36. The concept of the NM–protein corona is central in modeling the surface properties of nano-objects. It constitutes the primary nano-bio interface that experiences dynamic changes as the NMs move onto or into cells 36. NM surface features control biomolecules interaction with the particles and hence mediate their access to cells 37 eliciting or not an immune response.

To explore the influence of SV surface features on immunomodulatory aspects, we investigated the structure of polymeric vesicles, produced at a fixed ALG and CS concentration and mass ratio, focusing on polymer - polymer interactions. The impact of the synthesized ALG/CS SVs on a wide variety of human ex vivo immune subpopulations, T cells, B cells, Natural killer cells and monocytes was analyzed. Our approach allowed us to establish how specific chemical-physical characteristics of ALG/CS vesicles affect the immunocompatibility, regarding cellular uptake, viability and state of activation.

**EXPERIMENTAL SECTION**

**Vesicle Synthesis.** Microfluidic chip technology was exploited in order to synthesize natural polymer-based SVs for effective drug delivery. Briefly, ALG/CS SVs were obtained by two-step methods as recently reported 31: i) microfluidic aided ionotropic pre-gelation of alginic acid sodium salt (Sigma-Aldrich, product n. 180947, low viscosity, mannuronic acid to guluronic acid (M/G) ratio: 1.56) as Ca-ALG nanogels (NGs), followed by ii) bulk CS polyelectrolyte complexation. CS (Sigma-Aldrich, product n. 448877, medium MW: 190-310 kDa, deacetylation: 75-85 %) solution (1.2 mg/ml in acetic acid 1% v/v), was added dropwise to ALG nanogel suspension and bulk mixed under magnetic stirring for 2 h. After synthesis, ALG/CS complexed vesicles were collected by centrifugation. First, they were centrifuged at 800 rpm for 5 min to remove any large aggregates prior to analysis. Supernatant fraction is then resuspended in 15 ml of ultrapure water (UPW) (pH 6.5) and washed at 25°C in the Vivaspin Ultra centrifuge tube (100 kDa cut off (Sartorius)) two times for 30 min, at 4000 rpm, in order to remove acetic acid moieties and allow sample concentration.

**FT-IR measurements**. IR-spectra were acquired using a JASCO FT/IR6800 spectrometer coupled with an IRT-5200 microscope. Spectral manipulations were performed using the spectral analysis software from Jasco. Spectra were acquired in the range of 4000-650 cm-1 (resolution of 4 cm-1) at room temperature on a squared micro aperture of 100µm, with the accumulation of 100 repeated scans in reflectance mode. An isopropanol-treated silicon wafer was used as the background.

FT-IR analysis was carried out on the single polymer film (ALG or CS) and on bi-layer films (ALG/CS). For this purpose, ALG or CS was cast on a silicon slice and allowed to dry at room temperature; only for ALG film, a reticulation step was introduced pouring CaCl2 solution just few minutes after polymer casting. Bi-layer assembly was prepared by a two-step coating technique. ALG reticulated film, prepared likewise to the above procedure, was immersed in CS solution according to composite SV synthesis conditions.

**Vesicles Size and Zeta potential.** SVs were preliminarily incubated (at 37°C) in UPW or simulated blood fluid (SBF - RPMI, 10% FBS, pH~7.4), for 1 h or 24 h, in slow agitation on a 3D programmable mini-shaker (Biosan), to investigate their behavior in physiological conditions. After that, Vivaspin Ultra centrifuge tubes were used for SV washings (two times for 30 min, 25°C, at 4000 rpm), in order to remove medium residues not interacting with polymer molecules.

Dynamic light scattering (DLS) experiments were performed using a ZetaSizer Nano ZS90 (Malvern Instruments) equipped with a 10.0 mW He-Ne laser, operating at 633 nm, and an APD photodiode detector. For average hydrodynamic size and size distribution of bare- and surface-modified ALG NGs, as well as electrophoretic mobility measurements, SV samples were diluted 1:10 in UPW (pH 6.5), in disposable cuvettes (Malvern, DTS0012 and DTS1070) and monitored according to the manufacturer’s instructions.

For each SV sample, five measurements were performed in automatic mode at 37°C, in UPW. Cumulant analysis was employed for fitting of correlation curves, to determine mean translational diffusion coefficients, and the Stokes–Einstein equation was then utilized to convert diffusion coefficients to Z-average hydrodynamic diameter (HD). The mean average of peak positions in the intensity-weighted distribution of sizes was used to represent data from three independent measurements. SV electrophoretic mobility was converted to ζ-potential (ζP) values (mV) by the Smoluchowski’s approximation, and presented as the mean with standard deviation of three measurements.

**Human Peripheral Blood Mononuclear Cell Isolation and Cultur**e. Buffy coats were obtained from informed healthy donors (aged 25–50 years). Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat by Ficoll-Paque PLUS (GE Healthcare) density gradient centrifugation, based on methodology established by Bøyum 38.

PBMCs were cultured in RPMI 1640 medium (Gibco) containing 1% antibiotic-antimycotic mixture and 10% heat-inactivated fetal bovine serum (Invitrogen) in a humidified atmosphere of 5% CO2-95% air, at 37° C.

**Flow Cytometry Analysis.** The impact of ALG/CS SVs on PBMCs was monitored by flow cytometry analysis, in terms of SVs cellular uptake, viability and activation status. In all the flow cytometry experiments described below, the expres­sion of specific cell surface markers (clusters of differentiation, CD) allowed the identification of major immune cell populations of PBMCs. Specific CD molecules were detected by fluorescently labeled monoclonal antibodies (Bd Bioscence): (peridinin chlorophyll protein (PerCP)-anti CD3; allo­phycocyanin cyanin 7-conjugated (APC-H7)-anti CD14; phycoerythrin coupled to cyanin 7 (PECy7)-anti CD20; allo­phycocyanin (APC)-anti CD56).

After SV treatment, as described in details below, PBMCs were collected, stained by appropriate molecular mix, and then examined on a BD LSRII flow cytometer equipped with FACS Diva 6.0 software (Becton Dickinson, Germany). Data were analyzed with FlowJo v10 software (Tree Star, USA).

**Vesicle Uptake Analysis.** To quantify SV cellular uptake, fluorescent SVs were prepared by labelling the CS external layer of SV with the Abberion STAR 488 dye (Sigma-Aldrich), according to manufacturer’s protocol. Briefly, the NHS group of the dye was allowed to covalently bind CS amine groups, at pH 8. The reaction was conducted for 2 h at room temperature, in the dark. Three washings were then performed in UPW on the Vivaspin Ultra centrifuge tube (100 kDa cut off (Sartorius)) in order to remove free fluorescent dye traces.

PBMCs were transferred to a 6-well plate (1×106 cells/well) and incubated for 24 h in triplicate with labelled SVs at a concentration (3×109 SVs/μl) suitable for a cargo delivery, as shown in a previous functional study 32, or left untreated.

After that, PBMCs were collected and stained for 20 min by fluorescently conjugated monoclonal antibody mix and subsequently analyzed by flow cytometer. The mean fluorescence values of the positive cells internalizing SVs, obtained after proper gating, was considered to assess the level of SVs uptake per cell.

**PBMC Viability and Apoptosis Assay.** In order to detect cells undergoing apoptosis and necrosis, Annexin-V-fluorescein isothiocyanate (FITC, Invitrogen) and 7-amino actinomycin D (7-AAD, Invitrogen) staining was carried out. The assay is based on the binding of Annexin-V to phosphatidylserines (PS) on the extracellular side of membrane in early apoptotic cells, and it exploits 7-AAD ability to cross the membrane of dead cells.

PBMCs were transferred in a 12-well plate (5×105cells/well) and incubated in triplicate with SVs (3×109 SVs/μl), or sham-treated, for 6 h, 12 h and 24 h. Additionally, a positive control of cell damage was performed using a solution of 70% (v/v) ethanol (EtOH). The cells were then collected and stained with Annexin-V-FITC / 7-AAD and cell subset-specific antibodies, for 20 min in the dark. After washing, the cells were analyzed by flow cytometry.

**PBMC activation status after vesicle treatment, activation assay**. PBMCs were cultured in a 12-well plate (1×106cells/well) in the presence or in the absence of SVs (at the concentration of 3×109 SVs/μl) for 6 h, 12 h and 24 h, in triplicate; concanavalin A (ConA; Sigma-Aldrich 10 μg/ml) or bacterial endotoxin lipopolysaccharides (LPS; Sigma-Aldrich 2 μg/ml) were used as positive control. After that, for each time point, PBMCs were stained to identify immune cell populations and analyze activation marker expression (CD25, CD69 and CD80). Staining with fluorochrome-conju­gated monoclonal antibodies (mix I: PerCP-anti CD3 APC-H7-anti CD14, PECy7-anti CD20, APC-anti CD25, PE-anti CD69, FITC-anti CD80; mix II: FITC-anti CD3, APC-H7-anti CD25, PE-anti CD69, APC-anti CD56) was performed in the dark for 20 min. After washing, cells were analyzed by flow cytometry.

**Cytokine Assay.** Cell culture supernatants from PBMCs, after SV treatment, were used to spectrophotometrically quantify the secretion of tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) by ELISA kit (Boster Biological Technology), according to manufacturer’s protocol. ConA and LPS were used as positive controls. Briefly, cells culture supernatants were collected and diluted 1:10 using a sample buffer. Standards and test samples were added to 96-well plates pre-coated with monoclonal antibody specific for TNFα and IL-6 respectively. A biotinylated detection polyclonal antibody from goat specific for TNFα and IL-6 was added and then washed with PBS or TBS buffer. The HRP substrate TMB was used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acid stop solution. The density of yellow is proportional to the human TNFα and IL-6 amount of sample captured in plate. The absorbance was read at 450 nm using a microplate reader (Thermo Scientific).

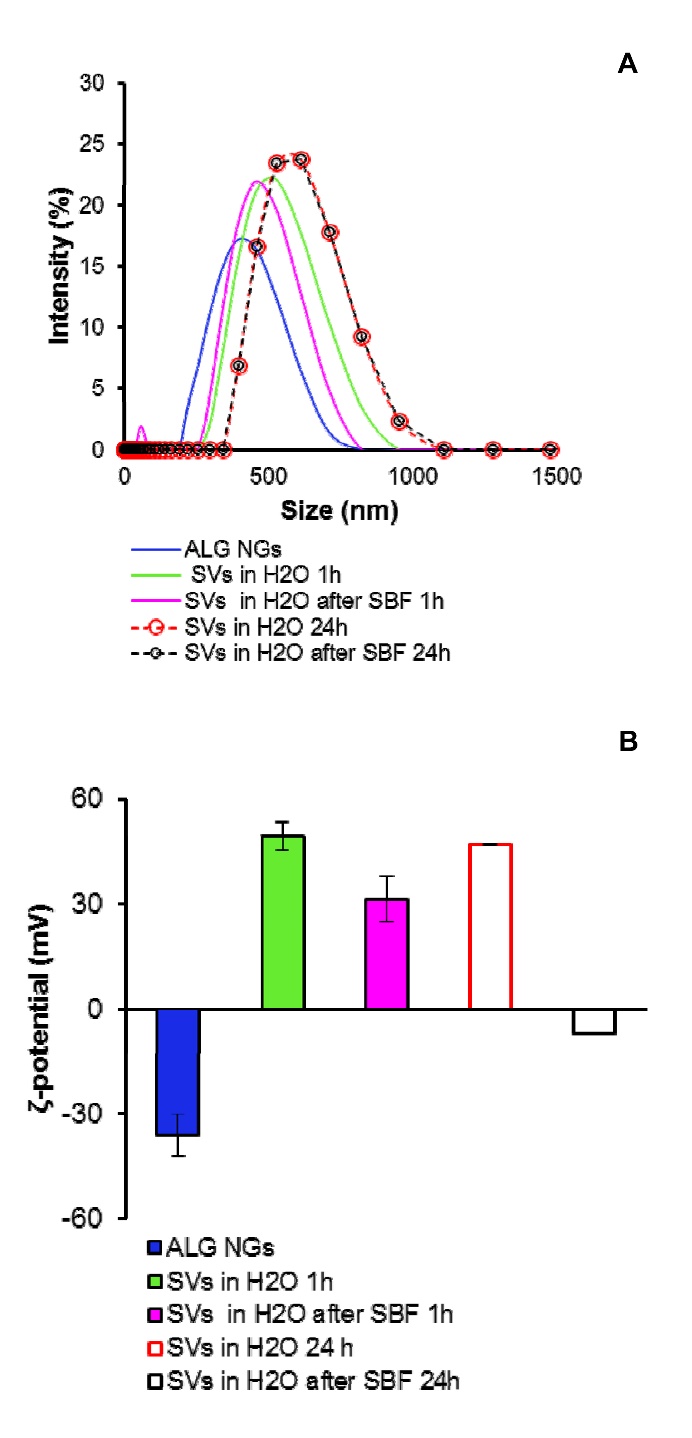
**Statistical Analysis.** Data analyses for FACS gated events were carried out using GraphPad Software Prism v5 (San Diego, USA). One-way ANOVA followed by Bonferroni’s multiple comparison test was applied. Data are presented as mean ± SD of technical replica (n=3); the differences were considered significant for p<0.05 and

**RESULTS AND DISCUSSION**

***Physicochemical characterizations.*** Physicochemical properties of NMs play a key role in their performance within biological milieu 36,39. Never the less, the microenvironment with which they come into contact affects the surface dynamics, which in turn determine the functional response (including kinetics, signaling, transport, accumulation, and toxicity) 40,41. Several reports underline that physical and chemical features of NMs regulate the interaction with the IS and, in details, surface electrostatic charges of engineered nanostructures may influence immune responses 42,43. Considering this, besides ALG/CS SVs HD and polydispersity index (PDI), we analyzed SVs in terms of ALG core - CS shell molecular interactions. FT-IR analysis and ζP measurements demonstrated that the experimental conditions set for SVs synthesis allowed the formation of a polyelectrolyte ALG/CS network.

***Vesicles size and zeta-potential****.* Measurement data, generated by Zetasizer software (version 9.01; Malvern Instruments Ltd.), indicated a mean SV HD of (620 ± 26) nm and a PDI of 0.518 ± 0.079 (in UPW) with the ALG NGs characterized by a mean HD of (456 ± 37) nm, PDI of 0.421 ± 0.051. After incubation in SBF (1h, 37°C, in slow agitation), results showed a double peak (PDI= 0.448 ± 0.029) (**Figure 1A**). The main peak at (474.0 ± 105.5) nm and the new smaller one at (58.91 ± 5.7) nm were most likely related to SV surface interaction dynamics with biomolecules, leading to the formation of a “protein corona” - that lowers the surface energy of the nanomaterial, and modifies its surface in terms of chemical composition and physico-chemical interactions 41,44,45. A slight CS external layer degradation is also expected, largely mediated by lysozyme digestion 46. When SVs were incubated in SBF for 24 h at the same temperature, the main peak was recorded at (526.4 ± 118.1) nm with a PDI of 0.418 ± 0.031. No smaller peaks were detected after 24 h in SBF, probably due to CS debris gradual enzymatic breakdown overtime. Protein interplay with SVs is subjected to a dynamic equilibrium process of binding and unbinding, owing to molecule abundance, molecule affinity and prolonged incubation time 41, thus reducing further lysozyme activity toward modified CS. Either at 1 h or at 24 h of SFB incubation, low aggregation of SVs, induced by protein adsorption, was recorded. In both cases, DLS analysis showed structures with micrometric size, in very small percentage compared the submicron ones (**Figure S2**).

SV ζP positive value ((+49.5 ± 4) mV)) was measured after complexation of CS-amino groups and the carboxylic acid groups of ALG which at low pH value (pH 5.3) provided negative charges to the ALG NGs, leading to ALG/CS SV assembly. In the presence of SBF (1 h of incubation), soft protein corona still allowed SVs to exhibit positive ζP value, even if reduced (+31.4 ± 6.58) mV, as reported in **Figure 1B**. Whereas, after incubation in SBF for 24 h, SVs displayed negatively charged surface, as demonstrated by their ζP value ((-7.11 ± 0.04) mV), due to the polymer-protein stronger interaction. Serum proteins have negative net charge at physiological pH thus SV cationic surfaces experienced a rapid and intense adsorption of high-affinity proteins upon exposure to SBF for longer time. ζP values are also consistent with previous reports, according to which, the ζP of CS-based carriers is significantly influenced by ionic strength and pH of the medium and strictly related to modification of the repulsive potential and the environment viscosity, which in turn influence chain conformations of CS molecules and subsequently the ALG/CS PEC complexes interaction 48,49.

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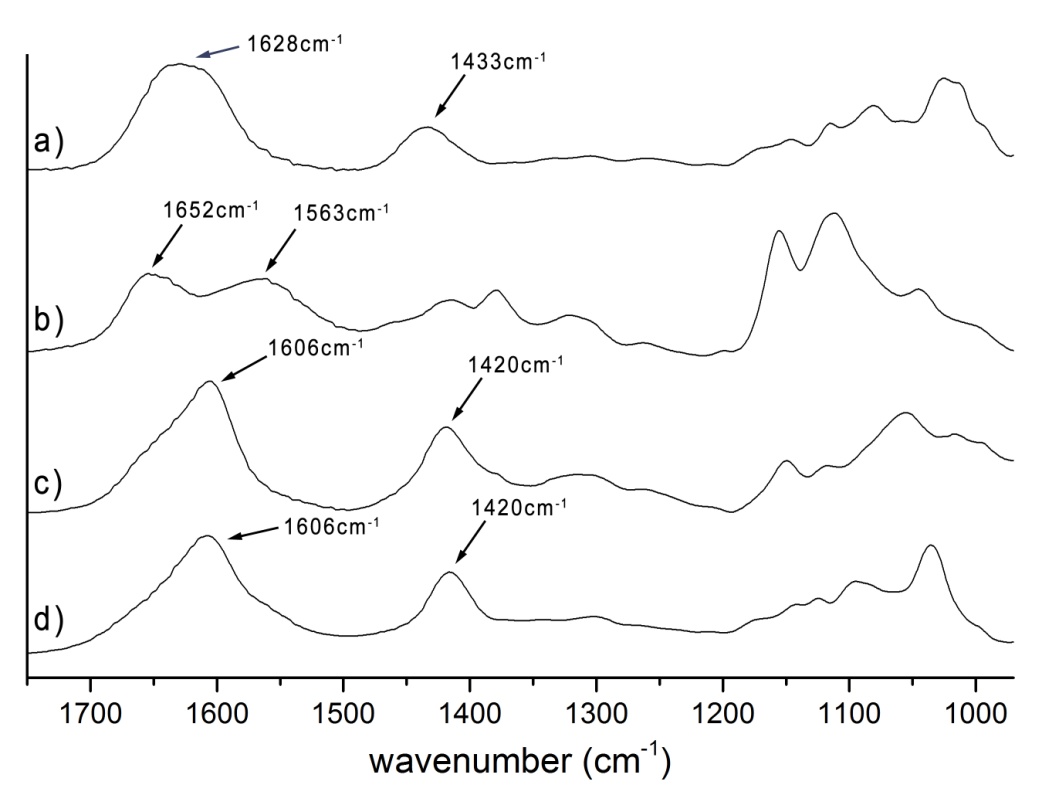
“**Figure 1. S**ize and surface chemistry characterization of nanoparticles. A) intensity-weighted size distribution of ALG NGs, ALG/CS SVs in UPW and ALG/CS SVs in SBF (1 and 24 h of incubation) obtained from DLS measurements. B) ζP mean values. Error bars represent three independent experiments.”

***FT-IR measurements.*** The alginic acid sodium salt (Na-ALG) was first dispersed in saline solution (NaCl 0.9% w/v, pH 5.3) and then reticulated with CaCl2. This treatment leads to a shift of the characteristic peaks of ALG that were recorded in the complex state at pH 5.3. The spectrum showed a broad band at 3480 cm-1 related to O–H stretching. The peaks at 1628 and 1433 cm-1 are associated, respectively, to the asymmetric and symmetric stretching vibrations of protonated carboxylate (**Figure 2a and S1a**). The IR spectrum of bare chitosan (dissolved in 1% acetic acid, pH 3.3) showed a broad band centered at about 3420 cm-1, related to the stretching vibrations from overlapping of the O–H and N–H bonds. The positive charged polysaccharide presents two characteristic absorption bands at 1652 and 1563 cm-1, corresponding to the C=O stretching (amide I) and N–H bending (amide II), respectively (**Figure 2b and S1b**).

To investigate the interaction between CS and Ca-ALG, we deposited two thin layers of polysaccharides, followed by washing steps with UPW, reproducing in a 2D configuration the steps applied in the production of colloidal PECs. After washings, the resulting pH of the polyplex film was assessed indirectly to point 6.4 by measuring the drained water.

The IR spectrum of the bilayer presented some changes in bands position and disappearance or appearance of new peaks in comparison to bare polysaccharides (**Figure 2c and S1c**). In the region 3200-3500 cm-1 a broader band was observed, due to the overlapping of peaks of –OH and –NH2. Below 1700 cm-1, we observed the appearance of a new peak at 1606 cm-1, not present in the single polysaccharide film spectra. Moreover, a less intense new peak was observed at 1420 cm-1. All the above -reported peaks in the region from single polysaccharide film disappeared. At the pH value used for ALG/CS complexation conditions (Na-ALG, pH 5.3; CS, pH 3.3) CS amine groups are protonated and electrostatic interactions occur between the two polymers, favouring the formation of ALG/CS SVs (CS pKa ~ 6.0–6.5; ALG pKa ~ 3.4–3.6 ) 50,51. We speculate that the -NH3+ (from CS), combined with a series of factors as pH and washing steps, drove a partial decomplexation of the carboxylate group of ALG from the Ca2+ ions. Thus, the described behavior could be attributed to the interaction of -NH3+ (from CS) with –COO– (from ALG), although it is not exhaustive to study this kind of interaction by FT-IR analysis 52. The interaction between CS and Ca-ALG was also confirmed from Ca-ALG NG and ALG/CS SV values of ζP, the latter showing a highly positive one ((+49.5± 4) mV), as results from the interaction of protonated amine with Ca-ALG NG surface (ζP (-36 ± 6) mV).

The two peaks at 1606 and 1420 cm-1 could be attributed to the asymmetric and symmetric stretching vibrations of carboxylate group and were revealed in the spectrum of Na-ALG before reticulation with CaCl2 (**Figure 2d and S1d**). Anyway, the electrostatic interaction of the –COO– with CS amine is weaker than the reticulation with calcium, therefore the carboxylate peaks of ALG where not shifted in the dual layered film. Also, it is noteworthy to consider that the 2D CS layer is much thinner than the ALG one, thus it was particularly difficult to detect the contribution of CS in the dual-layered film.



“**Figure 2.** IR spectra of Ca-ALG (a), CS (b), PEC bilayer films (c) and Na-ALG (d). The more descriptive portion of the spectra is between 1700 and 1000 cm-1, where the two polysaccharides show some characteristic peaks. In the PEC spectrum some new peaks appeared at 1606 cm-1 and at 1420 cm-1. These peaks were not observed in the individual films (a and b) but were revealed in non-reticulated Na-ALG spectrum (d). ”

**Vesicle uptake**

Notably, NMs with cationic surface exhibited high level of cellular uptake, in fact the positive charged surface will promote the internalization rate and further increase the internalization amount, compared with their negative counterparts, owing to the electrostatic interaction occurring with negatively charged cell membrane 17,53. In particular, CS-based nanosystems, showed significantly reduced circulation time and bioavailability, upon exposure to a biological environment, owing to their cationic surface 33,54. For these reasons, we first analyzed SV internalization trend by PBMC sub-populations (T, B, NK cells and monocytes) and then the influence of SVs on PBMCs, in terms of viability and function (by a comparative analysis of the most critical activation marker (CD25, CD69 and CD80) and cytokines (TNF-α and IL-6). We chose PBMCs as experimental model owing to their greater clinical relevance than other cell lines or specific populations. In this way, our ex vivo approach allowed us to perform assays and measurements directly on human cells, using experimental conditions which resemble in vivo context.

As reported in **Figure 3**, SVs were internalized in PBMCs after 24 h of treatment with differential behavior among the four PBMC major types of immune cells; the monocytes showed the greatest uptake of SVs. The 24-hour incubation time was adopted since this is a good time point to study the effect of any nanoparticle or NM on immune cells, as other research groups suggested 16, 55,56. In mammalian cells, five internalization routes are known: phagocytosis (via mannose receptor, complement receptor, Fcγ receptor, and scavenger receptor mediated pathways), macropinocytosis, clathrin-mediated, caveolin-mediated, and clathrin/caveolin-independent endocytosis 57,58. No receptor specific for CS has been yet identified in cell membrane; actually, it is thought that electrostatic forces allow interaction between the positively charged CS and the negatively charged cellular membranes, leading to internalization of polymeric complexes 59. Taking into account that T cells, B cells, NK cells are non-phagocytic cells, we assumed that ALG/CS SVs were preferentially internalized by monocytes (macrophage precursors) by interacting with negatively charged sialic acid groups exposed on monocytes surface 13, 60. According to literature, cationic or anionic and large particles are internalized more rapidly than smaller and neutral because they can be up-taken by macrophages via a mannose receptor-mediated phagocytic pathway 61,62.

Monocytes and macrophages possess broad homeostatic and surveillance functions and react against pathogenic particles, acting as scavengers and internalizing foreign invaders. Their ability to traffic through circulation and accumulate precisely at the diseased sites makes them an attractive tool for drug carriage and gene delivery 63-65. The cells can migrate into solid tumors and postulated to act as “Trojan horses” carrying lethal doses of drugs into tumors66. Particularly in the context of drug delivery, the SV internalization selectively by monocytes, without any immunomodulating action, is a very interesting result. In a recent study, Smith et al. demonstrated the selective uptake of single-walled carbon nanotubes by circulating monocytes for enhanced tumor delivery 67. Moreover, the preferential uptake of NMs can be useful in the context of blood disease in particular myelomonocytic leukemia 68.

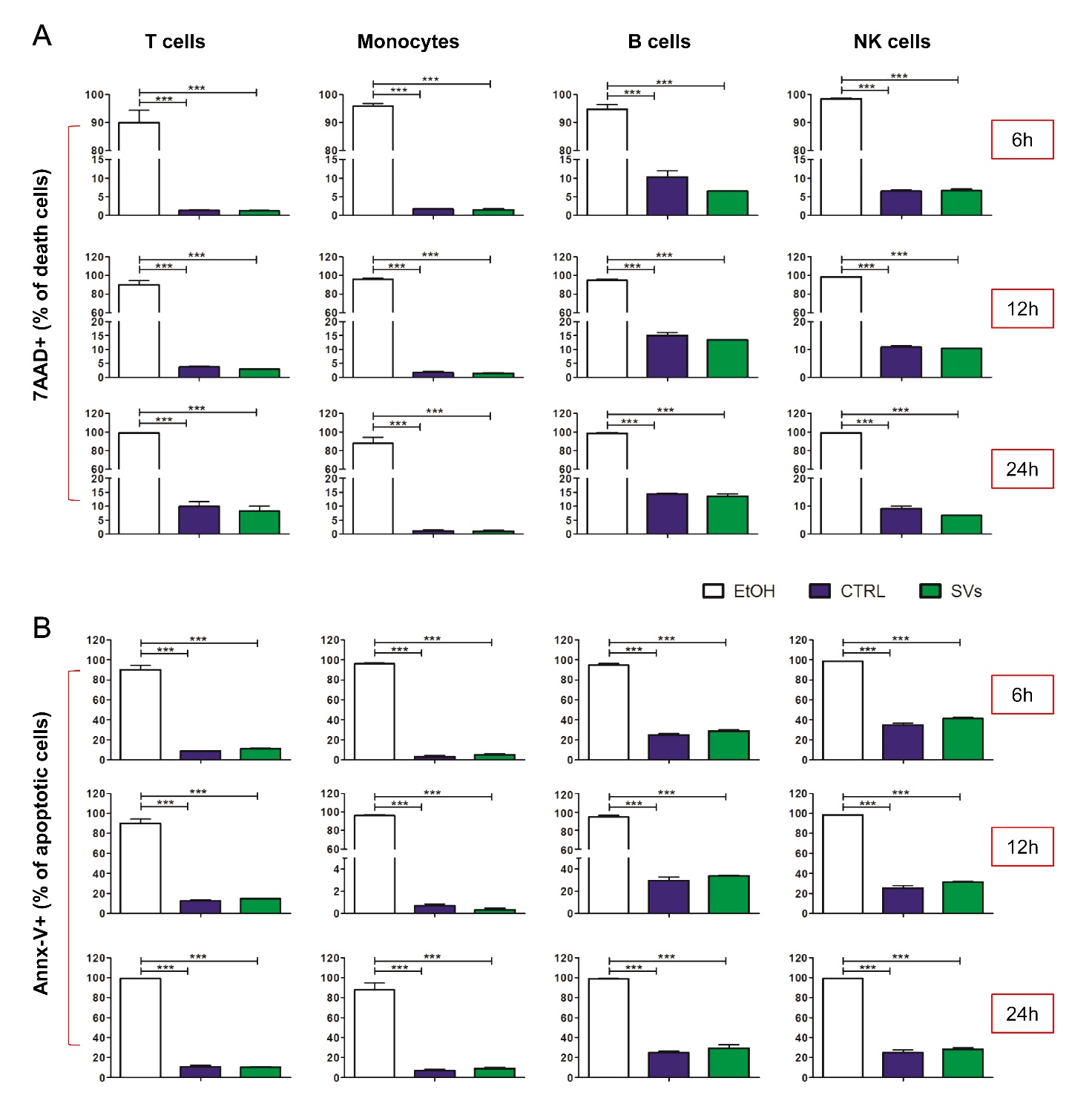
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“**Figure 3**. Uptake of SVs by human PBMCs. Peripheral blood mononuclear cells were either left untreated or incubated for 24 h with SVs. Samples were analyzed by flow cytometry as described above; data are expressed as fluorescence signal ratio (internalized SVs/Ctrl). Data refers to the mean values of three experiments (n=3). One-way ANOVA followed by Bonferroni post hoc test was used for statistical significance (\*\*\*p<0.001).”

**PBMC viability and apoptosis assay**.

Viability of PBMCs is a crucial point to study the impact of polymeric SVs on immune cells. In order to detect necrotic or early apoptotic PBMCs, we performed flow cytometry analysis by 7-AAD (**Figure 4 A**) and Annexin-V (**Figure 4B**) staining, after SV exposure at three different time points (6 h, 12 h and 24 h). As reported in **Figure 4A**, after SV exposure, the percentages of necrotic PBMCs were similar to negative controls (no significant statistical difference was recorded (p>0.05)).

Previous studies have established that cationic NPs are often more cytotoxic than anionic and neutral ones because they induce disruption of plasma-membrane integrity, which expose the negative charges of the phospholipidic groups, but also acidification of endosomes stronger mitochondrial, lysosomal damage, and increased number of autophagosomes 69,70. Remarkably, concerning the viability of PBMCs after SV treatment, our results demonstrated the high biocompatibility of SVs, evidencing the lack of toxicity on PBMCs. Levels of necrotic cells in SV-treated samples, monitored by 7-AAD staining, were similar to negative controls in each stipulated time points (6 h, 12 h, 24 h). Such findings were in agreement with previous studies reported in the literature: CS/ALG nanoparticles did not have a toxic effect on human monocytes, as demonstrated by MTT assay 71, and CS complexes did not affect the viability of murine macrophages 72. Annexin-V- staining highlighted that upon exposure to the SVs, no significant difference in the percentage of apoptotic cells was observed. Interesting, although monocytes were the PBMCs displaying the major uptake of SVs, only a slight increase of these Annexin-V+ cells was detected after treatment, with no statistical relevance (p>0.05) (**Figure 4B**). Our results were partially in agreement with a previous study in which CS-coated lipid nanocapsules were reported to affect cell apoptosis in T lymphocytes and monocytes 16.

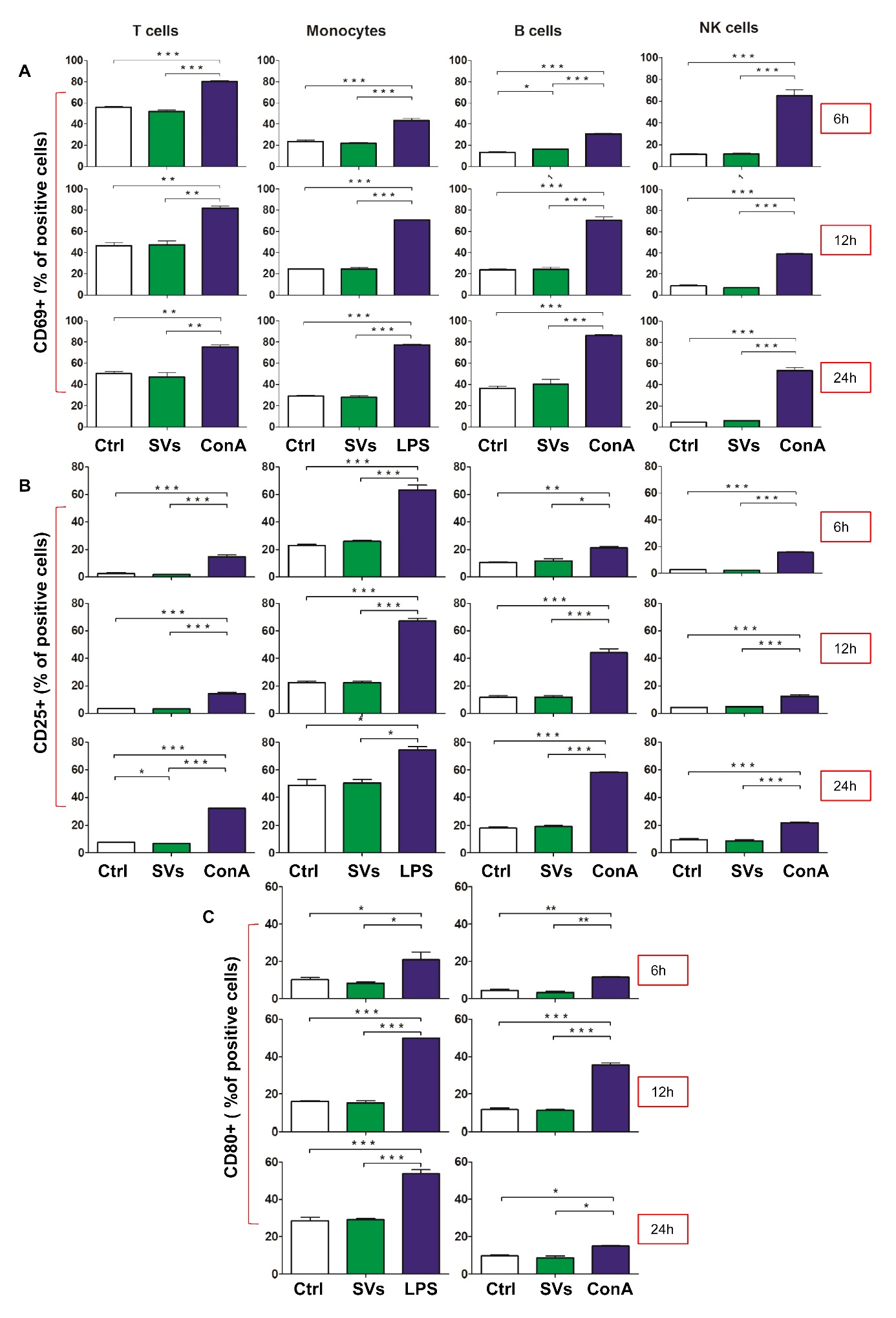


“**Figure 4. (A)** Viability of PBMCs after SV treatment, quantification of flow cytometry data. The viability of PBMCs was assessed after incubation with SVs. 7-AAD staining and cytofluorimetric analysis were performed after 6 h, 12 h and 24 h of incubation. EtOH was used as positive control. After SV exposure, no substantial necrotic process was observed on PBMCs compared to the vehicle control group (p>0.05). **(B)** Apoptosis assay on PBMCs after SV treatment, quantification of flow cytometry data. To investigate apoptosis, cells were incubated for 6 h, 12 h and 24 h with SVs or left untreated, stained with Annexin-V and with specific antibodies for cell population identification. Data refers to the mean values of three experiments (n=3). One-way ANOVA followed by Bonferroni post hoc test was used for statistical significance (\*\*\*p<0.001).”

**Activation status of PBMCs after vesicle treatment**.

**CD69, CD25 and CD80.** Possible immune activation mediated by ALG/CS SVs was investigated analyzing the expression of surface markers (CD69, CD25 and CD80), involved in the immune response activation process. CD69, a member of the C-type lectin superfamily, is one of the earliest cell surface antigens expressed by immune cells following activation; whereas, CD25 (alpha chain of the IL-2 receptor) is a late activation antigen; CD80 is expressed by monocytes and B lymphocytes, thus the analysis of its expression helps to monitor the activation of PBMCs populations and to determine how they, in turn, affect the reactivity of the others 16,73-76.

Interestingly, when PBMCs were incubated with SVs, CD69 expression did not reach statistical significance in T cells, monocytes and NK cells vs vehicle control group, evidencing no activation of these cell types. Of note, CD69 expression in B cells reached statistical significance at 6h (p<0.05) but not at 12-24h **(Figure 5A).** As for CD25 expression **(Figure 5B)**, we observed that SVs induced a significant increase of CD25+ T cells only after 24 h of incubation (p<0.05) and without any other sign of immune response, while the other PBMC populations did not show a significant difference between vehicle control group and SV-treated samples. The expression of CD80 **(Figure 5C)** did not show any significant difference in all PBMC subpopulations treated with SVs when compared with vehicle control group.



**“Figure 5**. **Impact of SVs on PBMC activation**. The activation status of human PBMCs was investigated into T cells, B cells, NK cells and monocytes, incubated with SVs for 6 h, 12 h and 24 h. Cell activation was assessed by evaluating the expression level of CD69 **(A)**, CD25 **(B)** and CD80 **(C)** markers. ConA and LPS were used as positive controls. CD25, CD69 and CD80 expression on different cell subsets were carried out in triplicate, and assessed by flow cytometry.” One-way ANOVA followed by Bonferroni post hoc test was used for statistical significance (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001).

Lymphocytes present low basal expression of CD69, which is rapidly enhanced following their activation 77. A transient activation-induced CD69 surface expression may be important for regulating T cell trafficking and differentiation, involving immunoregulatory cytokines 78. As reported in **Figure 5A**, we have found that CD69 expression on T cells, monocytes and NK cells was not affected by SV treatment; conversely CD69 expression in B cells reached statistical significance at 6h (p<0.05) and not at 12-24h **(Figure 5A)**. The induced activation of B cells by CD69 expression here reported is partially in agreement with the study reported by Borges et al. 79 in which the expression profile of CD69 in mice splenocytes was evaluated following exposure to the biopolymers, ALG or CS (administrated in solution forms - not as nanocomplexes) and other immunostimulatory factors. They showed that both polymers were able to upregulate expression of CD69 on B cells and CD4+ T-lymphocytes, with ALG as the least potent stimulus. Moreover, the expression of the CD69 molecule on CD8+ T-lymphocytes was observed only in splenocytes cultured with CS. Another recent study highlighted the role of NMs external coating, concerning the impact on immune cell response. In fact, Farace et al. demonstrated that CS-nanocapsules (CS-NCs) were able to activate T cells and monocytes, giving a powerful CD69 but also CD25 modulation on both immune cell types; conversely, PEG-NCs were completely inert 16.

**TNF-α and IL-6.** The immune-compatibility of SVs, demonstrated by viability and activation assays, was also confirmed by the recorded cytokines expression levels. SVs did not stimulate the release of TNF-α and IL-6 **(Figure 6)**, indicating that they did not enhance the innate immune response.

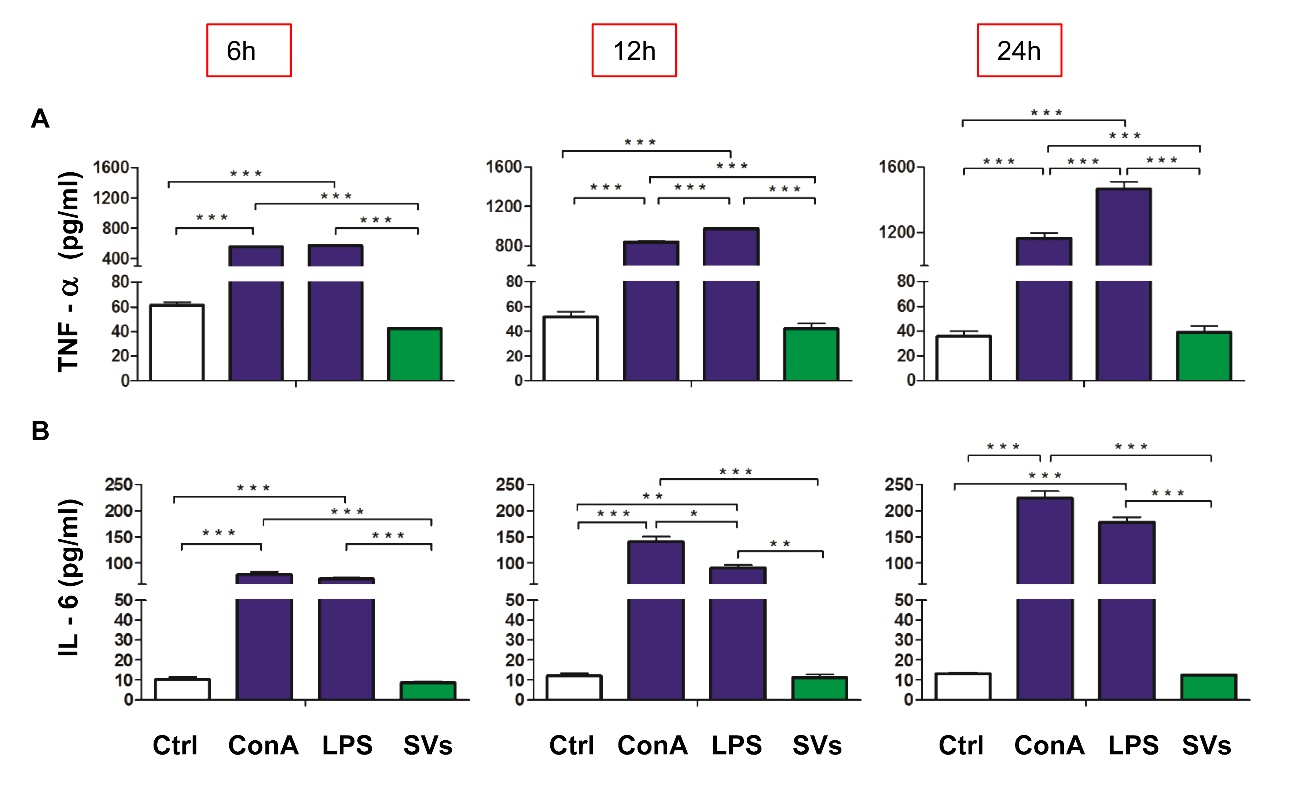
TNF-α is a potent cytokine normally secreted by monocytes/macrophages and its action is directed to the activation of the acute inflammation process 80. TNF-α activates leukocytes, stimulates fibroblast proliferation, promotes migration of inflammatory cells into the intercellular matrix, and triggers local secretion of other pro-inflammatory cytokines. IL-6, secreted by T cells and monocytes/macrophages, stimulate immune response leading to inflammation.

Orive et al. showed that the production of the pro-inflammatory cytokine TNF-α was approximately 100 times higher in the case of ALG based vaccine nanocarrier but these data were strictly related to purification grade of the polymer 81. In a recent study 82, the effects of CS on antigen presenting cells were analyzed: macrophages and dendritic cells were treated with CS derived from various sources, and the amount of TNF-α released by the cells was used as an indicator of immunoreactivity. They reported an increased level of TNF-α expression, and they indicated that only endotoxin content and not CS deacetylation degree or viscosity influenced CS-induced immune responses.

Démoulins et al. demonstrated that ALG-coated CS did not induce any detectable modulation of porcine or human blood dendrictic cells (DCs) and at the same time, they had little influence on the apoptosis levels and spontaneous cytokine profiles of DCs in culture 83. In another study, *Lactobacillus* induced the expression of IL-6 by monocytes and dendritic cells but its encapsulation in microcapsules of ALG/CS/ALG reduced the cytokines release suggesting anti-inflammatory role for ALG and CS polymers 84.

Results on the release of TNF-α and IL-6, together with those concerning the absence of toxicity and activation process after SV treatment, indicated good bio- and immune-compatibility of the synthetized structures with respect to the bare biopolymer based system.

CS derivatization has also been developed as strategy for new compounds in drug delivery applications 85,86, and possible development of ALG/CS SV in terms of polymer crosslinking and surface functionality are expected. Thus, additional examinations of the modified systems will be required with respect to the immunological behavior.

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**“Figure 6. (A) TNF-α secretion assay.** TNF-α release was assessed by ELISA on PBMCs. Cells were incubated with SVs for 6 h, 12 h and 24 h. After that, the supernatants were collected and analyzed in terms of TNF-α secretion level (expressed in pg mL−1)**. (B) IL-6 secretion assay.** PBMCs IL-6 release was assessed by ELISA. Cells were incubated with SVs for 6 h, 12 h and 24 h. After that, the supernatants were collected and analyzed in terms of IL-6 secretion level (expressed in pg mL−1). Data refers to the mean values of three experiments (n=3). One-way ANOVA followed by Bonferroni post hoc test was used for statistical significance (\*\*\*p<0.001; \*\*<0.01; \*p<0.05). No significant differences were recorded in SVs group vs vehicle control group (p>0.05).”

**CONCLUSIONS**

The need to explore immunotoxicological aspects of SVs arose from the fact that, although our systems were assembled by means of biodegradable and biocompatible polymers, the nature and strength of immune responses induced by ALG/CS NMs is still under-investigated in literature. In the present study, we synthesized submicron ALG/CS SVs and carefully evaluated their biological activities, strictly related to structural features, assessing their potential associated toxicity by ex vivo study on primary immune cells.

First of all, ALG/CS SV HD, polydispersity index (PDI), and ζP were monitored after incubation in UPW and in SBF (at 37 °C). Moreover, FT-IR characterization was performed on SV single building blocks (ALG and CS), as well as on polyelectrolyte complex configuration (ALG-CS PEC) in order to deeply investigate structural features of the resulting system before performing the immune profiling in terms of: i) SV internalization on PBMCs and subpopulations, namely T, B, NK cells and monocytes; ii) possible necrosis, apoptosis and proliferation of PBMCs, as descriptive of the adaptive and innate immune response; iii) the expression of the most critical activation marker, such as CD69, CD25 and CD80; iv) the production of cytokines (IL-6 and TNF-alfa) as effect on cell functionality.

We demonstrated that SVs have all the potential to be applied in the clinical practice (i.e. for systemic drug delivery) owing to green synthesis, biosafety of ALG/CS PEC and immunecompatibility properties. Sustainable production 87 and biocompatibility of polysaccharide PEC 88,89 are widely recognized. Conversely, there is still no clear consensus regarding their building block (as individual polymer) immunoreactivity 90,91, partly due to a lack of study standardization in polymer chemistry, source and purity 82. Although SVs were able to be in part uptaken by PBMCs, we reported that they were inert towards immune cells, and they showed a low immunomodulating activity. Remarkably, the absence of toxicity and activation *stimuli,* after SVs exposure, provides interesting proofs regarding the overall immune-compatibility of them as carriers92-94. These results gain particular value towards the biomedical applications of our SVs, and make these polymer-based structures more attractive for translation into pre-clinical and clinical investigations.

**Supporting Information.**

Experimental details of SV physicochemical characterization: alginate/chitosan film IR spectra; size measurements; absorbance and photoluminescence spectra of labelled SVs.

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**TOC FIGURE**

