University of Lisbon

Faculty of Pharmacy

Evaluation of *in vitro* immunological Passion Fruit-Like Nanoparticles for Cancer Immunotherapy Applications

Daniela Pavlicenco

Master in Pharmaceutical Sciences

2019

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Master Thesis in Pharmaceutical Sciences submitted to the Faculty of Pharmacy of the University of Lisbon

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2019

Abstract

The use of nanotechnology in medicine has expanded horizons of the therapeutic possibilities. One example is the use of nanotechnology for improving and developing new cancer treatments. Immunotherapy-based approaches with nanotechnology have emerged as a promising solution that could change cancer paradigm. Cancer immunotherapy employs different strategies to fight against cancer, such as monoclonal antibodies, immune checkpoint inhibitors, cytokines, cell-based immunotherapy and cancer vaccines. Nanovaccines are a product of the meeting between conventional vaccines and nanotechnology. In this work, passion fruits-like nanoparticles are evaluated for their toxicity and immunostimulation properties, in order to be further developed as a vaccine nanocarrier. The results suggest that pfNPs were nontoxic at 48 h up to 100 µg/mL for RAW 264.7 and KG-1 macrophages, as well as up to 10 µg/mL for BDCM cells. The pfNPs were highly uptaken by RAW 264.7, while their uptake was limited in KG-1 cells. The immunostimulatory properties of pfNPs were evaluated only in RAW 264.7 cells and showed a statistically significant increased expression of CD80 and CD86 receptors at 48h. These findings suggest that the pfNPs have a potential application as nanovaccines in cancer immunotherapy.

Resumo

O envolvimento da nanotecnologia na medicina abriu novos horizontes de possibilidades terapêuticas. Em particular, um dos exemplos é o uso da nanotecnologia para melhorar e/ou desenvolver novos tratamentos para o cancro. Neste contesto, as abordagens baseadas em imunoterapia junto à nanotecnologia surgiram como uma solução promissora que poderia mudar o paradigma do cancro. A imunoterapia emprega diversas estratégias no combate do cancro, tais como anticorpos monoclonais, *check point inibidores*, citocinas, imunoterapia baseada em células e vacinas terapêuticas. As nanovacinas são um produto da junção das vacinas convencionais com a nanotecnologia. No contesto deste trabalho, são avaliadas as propriedades toxicológicas e imunoestimulate das *Passion Fruits-Like* Nanopartículas, com o intuito de serem utilizadas posteriormente como nanotransportadores no desenvolvimento de vacina. Neste trabalho verificou-se que as pfNPs eram não tóxicas após 48h incubadas com nanopartículas de concentrações até 100 µg/mL para os macrófagos RAW 264.7 e KG-1, bem como concentrações até 10 µg/mL para as células BDCM. As pfNPs foram altamente capturadas pelas células RAW 264.7, enquanto que a captação pelas células KG-1 foi limitada. As propriedades imunoestimuladoras das pfNPs foram avaliadas apenas numa linhagem celular, RAW 264.7, mostrando um aumento estatisticamente significativo da expressão dos receptores CD80 e CD86 às 48h. Esses achados sugerem que as pfNPs têm uma aplicação potencial como nanovacinas na imunoterapia contra o cancro.

Acknowledgements

Foremost, I would like to express my sincere gratitude to my supervisors. To Prof. Helder A. Santos from the University of Helsinki for the internship opportunity in his group which made this project possible and reality. To Prof. João Pinto from the University of Lisbon for all his advice and support. I am sincerely grateful for all of this.

Special thanks to PhD Flavia Fontana for the continuous support, for her patience, enthusiasm, motivation, and immense knowledge. For her presence in all the time of my research and help in writing this thesis. I could not have imagined having a better advisor. Thank you, for awake in me the passion for the research.

Although this period of my life was filled with many ups and downs, this five years wouldn't have been possible without my friends, Monica, Sara and Viktoriya. Thank you so much, girls for your support and patience, I know I'm not a sweet cake.

The last and the most important, I would like to thank my family. To my parents, Vladimir and Eugenia for all their love, prayers, sacrifices, support and dedication in educating me for life. To my brother, Cristian who is still far away, helping to make my dreams come true and to my sister, Ana who has been supporting me and being on my side all my life. Thank you so much, you will always be a quiet haven for my wandering ship.

Abbreviations

ALL: Acute Lymphoblastic Leukaemia

APCs: Antigen Presenting Cells

CAR-T: Chimeric Antigen Receptor T Therapy

CPIs: Checkpoints Inhibitors

CT Antigens: Cancer Testis Antigens

CTLA-4: Cytotoxic T-Lymphocyte Antigen 4

CTLs: Cytotoxic T-Lymphocyte

DCs: Dendritic Cells

EDTA: Ethylenediamine Tetraacetic Acid

FDA: U.S. Food and Drug Administration

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

HBsAg: Hepatitis B Surface Antigen

HBV: Hepatitis B Virus

IL-2: Interleukin 2

IFN-α: Interferon-α

IRAEs: Immune Related Adverse Events

MDSCs: Myeloid-Derived Suppressor Cells

NKTs: Natural Killer T Cells

NP: Nanoparticle;

OECD: Organisation for Economic Co-operation and Development

OMVs: Outer Membrane Vesicles

OS: Overall Survival

PET: Positron Emission Tomography;

PD-1: Programmed Cell Death 1

PD-L1 Programmed Cell Death-Ligand 1

pfNPs: Passion Fruits-Like Nanoparticles

S1P: Sphingosine-1-Phosphate

SPECT: Single Photon Emission Computed Tomography

TAAs: Tumor Associated Antigens

TAM: Tumor-Associated Macrophages

TME: Tumor Microenvironment

TSAs: Tumor Specific Antigens

USAuNPs: Ultrasmall Gold Nanoparticles

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1. Pharmaceutical Nanoscience

Nanoscience refers to the study of matter, particles and structures on the nanometre scale(1). The increase interest in development of new nanostructures with specific properties and characteristics extend the application possibilities in nanomedicine. Pharmaceutical nanoscience has been growing considerably over the past 40 years(2). In the past, the formulation of drugs was limited to such pharmaceutical forms as solutions, compressed powders, encapsulated powders and oils, coarse suspensions and emulsions(3). Nowadays, due to the great advancement in science and technology, the horizon of conventional pharmaceutical forms is extended by nano-pharmaceutical dosage forms/nanomedicines(4). The pharmaceutical nanoscience approach is based on development of nanomaterials that can offer precisely engineered functions in the body(5). For example, nanoparticles (NPs) usually exhibit high surface-to-volume ratios, can have unique optical signals, specific shapes, surface modification, which can control the transport mechanism of various therapeutic cargo within the body both temporally and spatially(6).

Amongst the nanosized systems in pharmaceutical nanotechnology, the following systems have been widely reported: low molecular weight micelles(7), liposomes(8, 9), niosomes(10), solid lipid NPs (SLN™)(11), nanoemulsions(12), polymer-drug conjugates(13), polymersomes and filomicelles(14), polymeric NPs(15), porous silicon NPs(16, 17) and drug nanocrystals(18). However, new technologies are increasingly being developed in an attempt to fill in the blanks in theragostics applications which include the therapeutic and diagnostic strands(19).

2. Biomedical Applications of Nanomaterials

The terms nanomedicine has become mainstream for the application of nanoscale materials in medicine(20). Nanomedicine is at the interface between nanoscience, nanoengineering, and nanotechnology in an interdisciplinary field that is dedicated to engineering, diagnosing, treating, monitoring and preventing diseases(21, 22).

In diagnostic nanomedicine, nanomaterials are used as contrast agents for anatomical and functional imaging(23). For many diagnostic imaging techniques, such as magnetic resonance imaging (MRI), computed tomography (CT), radioactive imaging, such as Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT), photoacoustic imaging and fluorescence imaging, NPs are a key element(24). They can accumulate in specific tissues and increase the contrast, providing a better visualization of biological tissues(25).

In therapeutic nanomedicine NPs are used as a drug delivery system. They have been evaluated in several therapeutic areas, from cardiovascular diseases(26) to brain diseases(27). However, the most prominent disease focus has been in cancer(28, 29). The use of nanomedicine for cancer therapy has developed various strategies, such as: (i) chemotherapeutic-loaded NPs, where the cancer drug delivery to a solid tumor is determined by the efficiency in each step of this process - **c**irculation, **a**ccumulation, **p**enetration **i**nternalization and drug **r**elease (CAPIR); (30); (ii) NPs as delivery vehicles for antigens(31); (iii) NPs with stimuli-responsive release that can potentially reduce or alter the drugs' toxicity by their selective and specific release(32); and (iv) combining multiple antitumor drugs as alternative delivery strategies(33, 34).

Preventive nanomedicine includes applications like vaccines(35-37). Nanomedicine is actively used in vaccine formulation with the main purpose of enhancing immunostimulation, increasing effective delivery of antigens and ultimately improving the efficacy of vaccination(35). For example, Wang et al. demonstrated that positively charged nanogels (NG (+)) are preferable for Hepatitis B Surface Antigen (HBsAg) prophylactic vaccine carriers against Hepatitis B Virus (HBV), providing long-term protection(38). The development of vaccines using nanoscience has been approached not only for infectious diseases, but also for stimulation of immune system in cancer immunotherapy(39).

2.1 Cancer Immunotherapy

Cancer immunotherapy is a big area that focus is stimulating the immune system to prevent and treat cancer(40). Recently, it has become the standard approach for cancer therapy in different tumor types(41). According to the World Health Organization (WHO), in 2018, the global cancer burden was 18.1 million new cases and 9.6 million deaths(42). The application of novel immunotherapeutic agents dramatically changed the landscape of cancer treatment in recent years. Currently, many immunotherapeutic approaches are being developed and such results will help to define the expanding role of immunotherapy in cancer treatment in the future(43).

The extensive research into the mechanisms of immune activation and cancer-mediated immune evasion allowed the development of innovative methods to interfere, using exogenous immune mechanisms(44). Therefore, the activated immune system would be able to sustain cytolytic attacks against cancer cells, ideally resulting in total and permanent control over the tumor growth(45). However, issues emerge in cancer immunotherapy due to the evasiveness of the tumor cells(46). These cells are able to avoid the Natural Killer (NK) T cell responsible for immune surveillance through downregulating the ligands activating NK receptors(47) or through a deficient antigen presentation on major histocompatibility complex (MHC) class I/II molecules(48). The understanding of the behaviour of the immune system towards the “tumor system and microenvironment” is crucial for an effective and safe cancer therapy. For these reasons, multiple studies have been focused on the biological mechanisms to develop therapies that can counteract the tumor progression(49).

2.2.1 Mechanisms in Cancer Immunotherapy

The mechanisms of cancer immunotherapy can be represented by a cycle as shown in *Figure 1*. Due to multiple causes, cancer cells die by necrosis or apoptosis releasing tumor-specific antigens. These antigens are captured by antigen presenting cells (APCs), such as dendritic cells (DCs), which in turn present them on MHC. Mature APCs migrate to the lymph nodes, where they prime and activate the naïve T cells. Then, activated T cell migrate to and infiltrate the tumor site. There, they recognise cancer cells through interaction with T cell specific receptors, inducing cell death by apoptosis mechanism. This process increase cancer antigens in the bloodstream, strengthening the immune response(41). These mechanisms are extremely important for the creation of anti-cancer immunity. However, these natural mechanisms most often fail, inducing the progression of the disease.

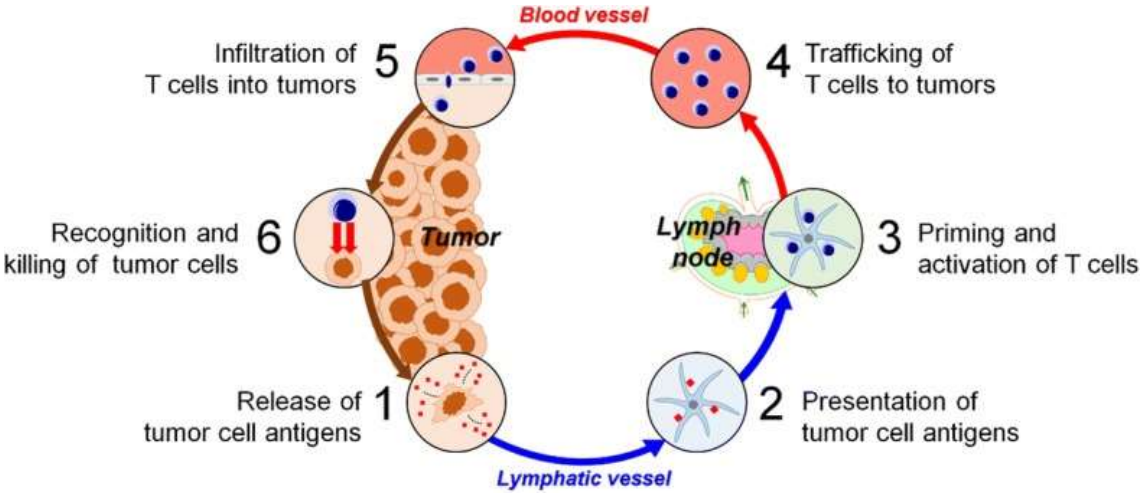


Figure 1 Cancer immunity-cycle. [1] Cancer cells release the specific antigens when they die, [2] Capturing and presenting cancer-antigens by APCs to T cells, [3] activation and proliferation of immature T cells, [4] migration of T cells and [5] their infiltration to the tumor site, [6] Recognising and killing the cancer cells. (reproduced from Ref. (41), copyright 2018, The author(s) under a Creative Common Attribution 4.0 International License)

This cancer-immune system cycle shows how the immune system should work in normal conditions. However, during tumor development, cancer cells become able to evade the immune system through the induction of immune tolerance which consist in turning off the tumor cell antigens(50). The second process is known as immune evasion(51), which occurs when the tumor creates a different environment around itself, known as a tumor microenvironment (TME), which protects it from the immune antitumor response(45, 52).

Amongst the different negative players in the TME, macrophages, in response to specific signals, can be activated in the M1 or M2-type functional phenotypes. M1 is a proinflammatory macrophage, with the ability to kill tumor cells and pathogens by stimulating inducible nitric oxide synthase, with the production of nitric oxide and tumor necrosis factor α(53). In contrast, M2 is a heal-type macrophage which typically express arginase-1(54, 55). After the activation of M1-polarized macrophages, dead cells release immunosuppressive factors such as IL-10, TGF-β, and S1P. Those factors stimulate the macrophages to repolarize from M1 to M2(53, 55). Furthermore, because of the chemoattractants secreted by tumor cells, the monocytes infiltrating into the TME differentiate in tumor-associated macrophages (TAMs)(56). TAMs are associated with progressive tumor cell growth and invasion of tissues(57). In addition, infiltration of myeloid-derived suppressor cells (MDSCs) after tumor cells death contributes to the suppression of the immune response(41, 58). MDSCs are a heterogeneous population of immature myeloid cells characterized by the ability to suppress T cell proliferation and cytotoxicity, inhibit the activation of NK T cells, and induce the differentiation of immunosuppressive regulatory T cells (Treg). It has been shown that MDSC levels

correlate negatively with the prognosis and survival of patients with cancers in various stages and types(58).

Another mechanism that allows cancer cell to avoid the immune response is related with the expression of suppressive molecules on cancer cells such as programmed cell death ligand 1 (PD-L1) and programmed cell death ligand 2 (PD-L2) and on T cells, such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death 1 protein (PD-1)(59). These molecules supress the activation of Cytotoxic T-Lymphocyte (CTLs)(60). However, the research into therapies to interfere with these signals as a strategy for immunotherapy is running since the 1990's(61). Nowadays, the intervention on this mechanism is considered a successful clinical strategy used in cancer immunotherapy(62).

2.2.2 Current Strategies in Cancer Immunotherapy

Currently, five key immunotherapeutic modalities have been approved for clinical use(52). These can be classified into two general categories: passive and active immunotherapies. Moreover, they can be employed individually or in combination(63). On one hand, the passive approach for immunotherapy involves the use of monoclonal antibodies, lymphocytes and cytokines produced externally. On the other hand, the active approach focuses on enabling the host's immune system to recognize the tumor antigens on the cancer cells surface (44, 52). It is noteworthy that the effectiveness of immunotherapy is not homogeneous. It is highly dependent on the type of cancer, stage and intraindividual patient response(64).

2.2.2.1 Monoclonal Antibodies

The aim of a monoclonal antibody is to recognise and target a specific tumor associated antigen (TAA). This strategy of immunotherapy has been used to treat many different types of cancers(65).

The mode of action of monoclonal antibodies requires that one antibody can recognise one specific sequence or epitope(66). Anticancer monoclonal antibodies are made to recognize an antigen expressed only on cancer cells or an antigen that is overexpressed in these cells(67). Despite these monoclonal antibodies being so specific, the therapeutic response can vary(68). Firstly, if the antigen is specific of cancer cell, it also needs to be expressed on the surface of cells(69). However, even a smaller mutation of the gene that codifies for the antigen can alter the epitope recognised by the antibody, making the therapy inefficient(70). Secondly, if the antigen is just overexpressed in cancer cells, but still expressed also on healthy cells, the therapeutic

antibody will induce cell death not only on tumor cells, but also in healthy cells, increasing side effects(44, 52).

Currently, antibodies for cancer immunotherapy can be unconjugated and exert a cytotoxic effect on tumor cell by themselves or conjugated to a drug increasing the drug's specificity(71).

2.2.2.2 Immune Checkpoint Inhibitors

Inhibitory immune checkpoints are very important for a physiological balance since they maintain self-tolerance and attenuate the overreaction of immune system in the presence of pathogens, avoiding tissue damage(72). Tumor cells take advantage of this strategy, expressing such immune-inhibitory molecules to induce tolerance(73).

Increasingly, new immune checkpoints expressed by the tumor cell have been discovered(74). However, the most frequently used are the monoclonal antibodies that target PD-1, PD-L1 or CTLA4(73). For their discoveries and contributes in cancer immunotherapy on the inhibition of negative immune regulation, Drs. James P. Allison and Tasuku Honjo were awarded with the Nobel Prize 2018 in Medicine and Physiology(75). At the same time, Dr. Allison and Honjo developed different strategies that unravelled the mechanisms of immune cell responses to cancer, specifically the inhibition of brakes on the immune system for the same end goal – cancer treatment(76, 77).

Particularly, Dr. Allison and his research group were the first to discovered the protein CTLA-4, an immune checkpoint receptor expressed on the surface of activated T cells(78). His group created an antibody that could bind to CTLA-4 and block its function removing the central immune tolerance and reinstating the anti-tumor immune response (79). The CTLA-4 receptor prevents the early activation of naive and memory T cells by interactions with its ligands B7-1 (CD80) and B7-2 (CD86)(80). After the successful *in vivo* experiments in rodents(81, 82), the 2010 clinical studies showed great results in patients with advanced melanoma(83) and prostate cancer(84). The research in this area has led to further improvements and to the extension of the application to other tumor types (85-88).

Almost simultaneously, Dr. Honjo and his group discovered the protein PD-1 on the surface of immune cells and, after studying its function, revealed that it also act as a brake, similarly to CTLA-4, but with a different mechanism of action(89). PD-1 is a receptor expressed on the surface of many cells such as activated mature T cells, activated NK cells, B cells, monocytes and various normal tissues cells, playing a

predominant role to maintain peripheral tolerance through its interaction with PD-L1 and PD-L2(90, 91).

Later, during *in vivo* studies, PD-1 blockade by monoclonal antibodies was shown to be a promising strategy in cancer therapy(92-94). Moving to clinical trials, a breakthrough in therapeutic efficacy in different types of cancer has been demonstrated(95). The results achieved long-term remission and a possible cure in various patients with metastatic cancer (96-98). Currently, immune suppressive blockers such as anti-CTLA-4 and anti-PD-1 monoclonal antibodies have been approved by U.S. Food and Drug Administration (FDA) for the treatment of different tumor types(99). Among those approved, there are Ipilimumab(100, 101), Tremelimumab(102) (CTLA-4 blocker) and Pembrolizumab(103-105) (PD-1 blocker).

To recognize and eliminate tumor cells, CTLs require more than one activating signal. In the early immune response, CD8+ T cells recognize an antigenic tumor peptide presented by the MHC class I on the surface of cancer cells or by APC (first signal). The second signal, called "co-stimulatory signal" completes primary activation through binding of the T cell CD28 receptor with two co-stimulatory ligands expressed on APCs; B7-1 and B7-2(106, 107). Then, T cell activation occurs, followed by further differentiation into CTLs expressing on the surface specific receptors such as CTLA-4 and PD-1.

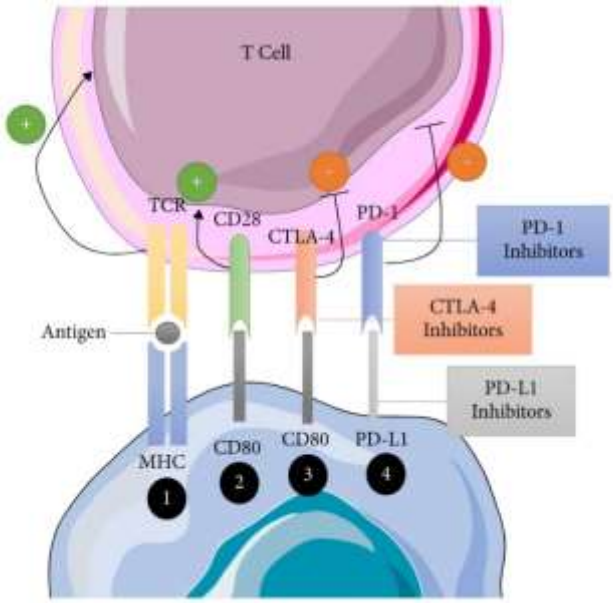


Figure 2 Mechanism of immune checkpoint inhibition (Reproduced with the permission from Ref. (52), copyright 2019, Manpreet Sambi et al. under the Creative Commons Attribution License)

Under physiological conditions, when CTLA-4 establishes a bond with B7-1 or B7-2 it prevents T cell from overreacting, acting as a negative regulator of T cell activation, while keeping the balance between inactivity and activity of CTL(80).

Similarly, the engagement of PD-1 on a T cell with PD-L1 on the tumor cell surface attenuates T cell function and activation. However, in terms of toxicity, it was expected that PD-1/PD-L1 blockers would have a better anticancer effect than CTLA-4 blockers and less side effects due to the level of the immunological synapsis involved (tissue *vs.* systemic) (108, 109).

However, given their importance in maintaining self-tolerance, the inhibition of these pathway can result in immune related adverse events (IRAEs)(110). IRAEs can induce toxicity and cause tissues and organs damage, leading to autoimmune-like diseases and inflammation. IRAEs are less frequent with anti-PD-1 blocker than with anti-CTLA-4 ones (13.3% as opposed to 19.9% in anti-CTLA-4) (111). For this reason, anti-PD-1 treatment is approved as first line for advanced melanoma in the USA and the EU(112). Another strategy to reduce the side effects is strategically combine different types of checkpoint-blockers reducing dosage of each component without compromising efficacy(113).

2.2.2.3 Cytokines

Cytokines are molecular mediators responsible for intracellular communication via shared receptors where they act by enhancing or inhibiting the effector cellular protein components that regulate the function and homeostasis of the immune system(45). The generation of efficient antitumor immunity requires, on one hand, a variety of cytokines that positively regulate the functions of antigen-specific effector cells to recognize and reject tumor cells and, on the other hand, the suppression of mechanisms that allow tumors to escape immunologic detection(52). As a result of the important role that cytokines are playing on the regulation of antigen presenting cells and activity of T cells, they have been investigated as a potential strategy in cancer immunotherapy.

Currently, two types of cytokines, Interferon-α (IFN-α) and Interleukin 2 (IL-2), are used for cancer treatment(114). An important requirement, influencing the outcome of the therapy, for the treatment with these cytokines is the presence of a relatively robust immune system in the patient(115). The use of IL-2 in cancer immunotherapy increases the antitumor activity through the activation of CD4+ and CD8+ T cells(114), NK cell and specific tumor-infiltrating cells(45, 52) Similarly, IFN-α has been characterized as an immune stimulator. IFN-α contributes to the activation of DC and of the antigen presenting mechanisms, enhancing Th1 cells response, CTL and NK cells activity. IFN-α can be administered in combination with cancer vaccines to enhance their therapeutic efficacy or with other immunotherapy treatments(52, 114).

2.2.2.4 Cell-Based Immunotherapy

Amongst the different types of cell-based immunotherapies employing T cells the first treatment consists in transferring to a patient natural or genetically-modified T cells, which have been expanded *ex vivo* to recognise tumor antigens and kill the cells (116). Another type of cell-based immunotherapy employs NK cells. NK cells are lymphoid cells responsible for innate immunity that provide early protection against cancer development and metastasis(117-119) characterised by high selectivity to kill abnormal cells without any need of prior stimulation(120). NK cell dysfunction usually is associated with cancer development and bad prognosis. Therefore, the re-establishment of endogenous NK cells or transferring of NK cells with improved function promise potential efficacy in cancer treatment. In addition, cell lysis induced by NK cells is supplemented by the lysis triggered by cytotoxic T cells, thereby NK cells are considered as an alternative or complementary therapeutic for cancers that are refractory to T-cell based immunotherapy(120).

Furthermore, another strategy in adoptive cell-based immunotherapy is the use of cytokine-induced killer (CIK) cells, which are a heterogeneous subset of polyclonal T-effector cells with both NK and T-cell properties(121). In the beginning, clinical studies on CIK cell therapy have provided promising results and this therapy has moved from ‘bench to bedside’. However, problems, such as inadequate quality control of CIK cells, have led some of the countries to stop CIK cell therapy(122).

Finally, the most promising cell-based immunotherapy is chimeric antigen receptor (CAR)-T cells therapy. This therapy integrate the antibody specificity together with the cytotoxic and memory functions of T cells (123). CAR are artificially engineered receptors, can be designed to be less susceptible to negative regulation by incorporating costimulatory signals in their structure, overcoming cancer-mediated immune tolerance(124). In theory, CAR-T cells could express those receptors on their cell surface with non-Human Leukocyte Antigen [HLA]-restricted tumor antigens, activating T cells and target them to malign cells(125).

The process of production of CAR-T cells for the clinical practice starts with the isolation of mononuclear cells from peripheral blood. This step is followed by stimulation of T cells via monoclonal antibodies or cytokines. Then, the transgene encoding CAR mRNA is transfected to the T cell through viral or non-viral approaches, in clinical trials usually via retroviral vectors(126). After transduction, the genetically modified T cells are

cultured to increase their concentration and, after a two-week period, they are injected back to the patients(127, 128).

With the advance of medicines, the chemotherapy has improved their response rates in adult and paediatric patients with T cell Leukemia up to 90%(129-131). However, in two-thirds of these patients, the chemotherapy continued with limited success for relapse of cancer(132, 133) and the alternative options for them are poor. In this tumor type, CAR T cells therapy has revealed its extreme importance producing remarkable response rates(134).

The conventional therapeutically approach for patients with B-lymphoid malignancies chemotherapy has been associated with high toxicity and insufficient efficacy (135). Due to several similarities shared between T-line malignancies and B-line malignancies, CAR T cells therapy can be employed in both(136). This therapy has shown remarkable results in B-line malignancies. Particularly, in Acute Lymphoblastic Leukemia (ALL) of B cell, the results were unprecedented with up to 90% of complete remission rate using anti-CD19 CAR-T cells(136, 137). However, the efficacy of CAR-T cells in solid tumors has been low. The reason for this unsuccess could be related to the specific characteristics of TME(137, 138).

Currently, the genetically engineered CAR-T cells targeting CD19-Positive ALL-B cells became the first licenced T cell therapy for cancer being approved by FDA in 2017 for paediatric and young adult patients(139-141).

2.2.2.5 Cancer Vaccines

In recent years therapeutic cancer vaccines have gained great popularity as emerging new approaches for specific oncologic indications(142). This strategy involves conventional vaccination methods to stimulate the immune system to fight against cancer(143). In the development of vaccines the whole or fragments of cancer cells or specific immunogenic antigens can be used to promote the activation of immune system(144). The mode of action after immunostimulation is shown in *Figure 3*.

The specific tumor antigens are uptaken by DCs, then are processed in the Golgi bodies and presented to helper T cells via MHC II(145). The activated CD4+ T cells secret IL-2 cytokine which consequently activates other cells such as cytotoxic T cells and NK cells(146-149).

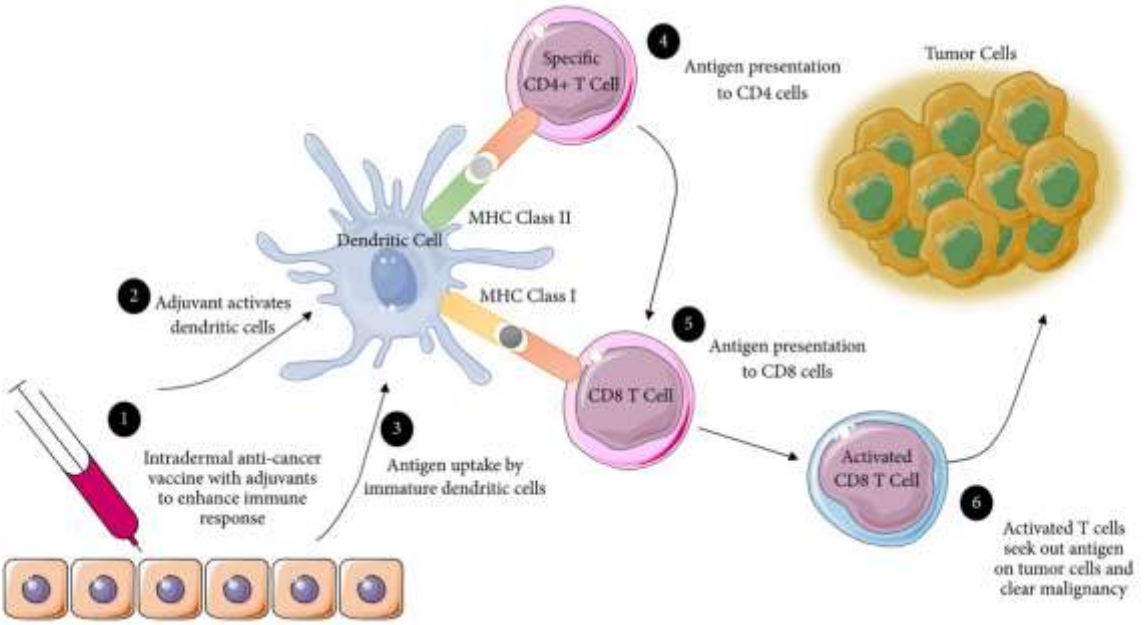


Figure 3 Vaccines for cancer treatment. The figure represents the events happening after intradermal vaccine administration [1] together with the adjuvants that promote dendritic cells activation [2]. The specific tumor antigens are up taken by the APC (DC) cells [3], later processed and presented to the immune cells such as CD4 cells [4] and CD8 cells [5] via MHC I and II. Consequently, CD8 cells are activated to seek out the antigen on the surface of cancer cells [6]. (Reproduced with the permission from Ref. (52), copyright 2019, Manpreet Sambi et al. under the Creative Commons Attribution License).

Firstly, after activation, a CTL-based antitumor immune response is generated(150). Secondly, memory immune cells that prevent relapses and metastasis of cancer are primed(151). Despite the theoretical premises, the clinical efficacy of vaccines is variable amongst different patients(152). One of the biggest factor contributing to these variations is the specificity of the cancer vaccine to individual immune system, which cannot be generalized(153). The correlation between the responses could be better if the vaccine would be formulated with the patient's own cancer antigen, however, even there, differences in rate responses would be expected due to the stage of immune suppression in each tumor lesion in each patient(154).

2.2.2.5.1 Vaccine antigens

One of the most important steps in cancer vaccine design is the choice of antigen. Ideally, the antigen should meet the following criteria [1]: it should be expressed only by cancer cells, [2] presence on every cancer cell surface so that they can be recognised by immune system, [3] being necessary for cancer cell survival such that downregulation of the antigen induce cell death, and finally [4], it should be highly immunogenic to activate the immune system(155). Almost none antigen meets these criteria(156).

Tumor antigens can be classified into two classes, tumor associated antigens (TAAs) and tumor specific antigens (TSAs). The first class includes so-called cancer testis (CT antigens) also known as cancer germline antigens(157); antigens that are

overexpressed in cancer cell and antigens expressed only during cell lineage differentiation(155). The second class, TSAs include the oncoviral antigens and neoantigens which can be common among the patients (shared) or specific of the patients (private/personal) (155) (*Figure 4*).

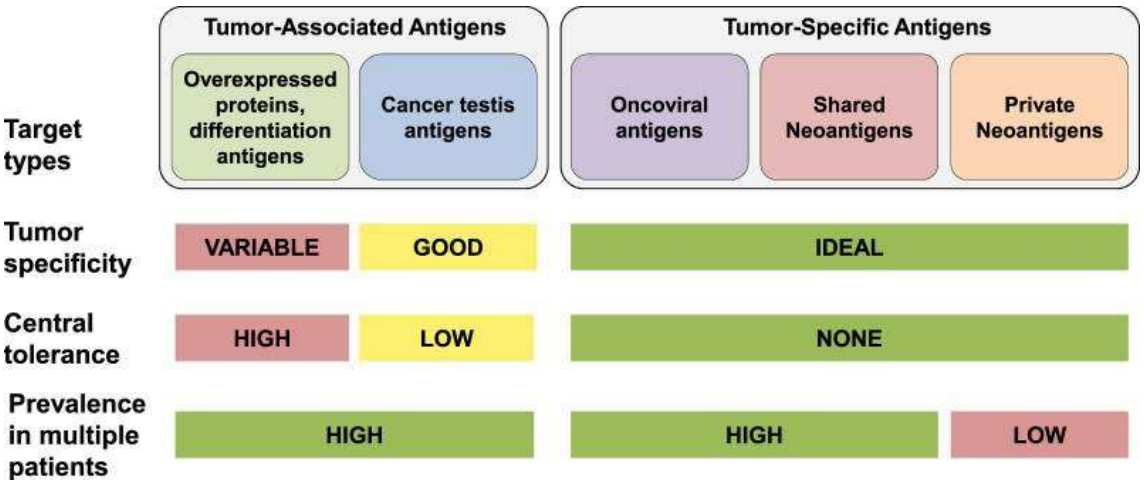


Figure 4 Schematic of two general classes of antigens: tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs). Each general class is divided into sub-classes related with targeting types referring to their tumor specificity, central tolerance and prevalence in multiple patients. (Reproduced with the permission from Ref. (155), copyright 2019, The author(s) under a Creative Common Attribution 4.0 International License.)

TAAs are self-antigens that are abnormally or preferentially expressed in cancer cells(158). However, at some level, they can also be expressed in normal cells(159). Nowadays, most of the developed vaccines are for the targeting of TAA, since they are easier to identify and present a high prevalence in multiple patients. The development of vaccines against TAAs is hindered by several barriers(160). One of them is the presence of central and peripheral tolerance, mechanisms employed by the immune system to remove B and T cells with high affinity to self-antigens (including TAAs). Thus, TAA-targeting cancer vaccines should break the tolerance by activating the low-affinity T cells or any remaining TAA-reactive T cells(161). To amplify the activation and expansion of TAAs-reactive T cells, the strategies employed so far have been using addition of potent adjuvants, co-stimulators, and re-vaccination (162). Despite all the modifications, clinical trials show that the immune response is not strong enough to present a significant efficacy(158). Another challenge for targeting TAAs vaccines is the collateral damage caused in normal cells that also express the same self-antigen.

TSAs are considered truly tumor-specific(163). They include the antigens expressed by oncoviruses, as in case of cervical cancer induced by HPV E6 and E7(164). Oncoviral antigens are highly immunogenic, therefore, vaccines targeting these antigens have been tested to be effective when are used in prophylaxis; however they

are not effective in treating already established cancer(153). Additionally, TSAs include so-called neoantigens(165). They are encoded by genes derived from mutations, peptides that are completely absent from the normal human genome presenting an altered protein sequence(166). Neoantigens can be divided in two types: shared neoantigens, when they are prevalent across patients/tumor types; and private neoantigens, that are unique to individual patients' tumors(167). The last ones are the majority, and therefore require a shift towards more personalized therapy. As a result of their specificity, they are not subject to central and peripheral tolerance mechanisms(168). Data from numerous preclinical models using vaccine based on targeting neoantigens have clearly shown defined anti-tumor immunity(169). Moreover, they have been tested in early (Phase I) human clinical trials for melanoma (different stages) whit very promising results(167, 170).

2.2.2.5.2 Vaccine vectors

To formulate an effective vaccine, it is not enough to have a good antigen(171). If the vaccine is delivered incorrectly, it will not work well(172). The choice of vaccine formulation is critical to release the antigen and/or stimulate the immune system correctly(173). Currently, different vaccines-based approaches are in different stages of development(174). In general, five types of vaccine platforms are being developed for cancer therapy: cellular vaccines; peptide vaccines; viral vector vaccines engineered to express TA; DNA and RNA vaccines; and biomaterial-assisted vaccines(175, 176).

The cellular source in the case of cell-based vaccine can be various. Autologous APCs loaded with tumor antigens have demonstrated some efficacy in patients(177). An example of this approach is Sipuleucel-T(178). This therapy has demonstrated a survival advantage in patients with asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer(179). Sipuleucel-T was the first autologous cellular immunotherapy approved by the FDA(180). DC vaccines have also been studied. They are either loaded with peptide antigen or transfected with antigen genes. Some studies show a small increase in median overall survival (OS) (25.8 vs 21.7 months for placebo) with a good toxicity profile(181). Vaccines composed by whole tumor cells that are genetically modified to secrete immune stimulatory cytokine such as GM-CSF cytokine can stimulate antigen presentation, activation, and survival of DCs(182).

Synthetic peptide-based cancer vaccines are safe, well-tolerated and able to specifically stimulate T cells (*Figure 5*). However, despite all the efforts, clinical trials have not shown a good immune response and significant benefit has been elusive(183). Earlier, single short peptides have often been used, which in some cases were not able

to overcome antigen heterogeneity or stimulate a good immune response(184). Short peptides (<15 amino acids) can be presented to T cells by many nucleated cells since they do not require processing by APCs. However, the antigen presentation processed by other cells than APCs does not provide a proper binding between the epitopes and receptors, which do not promote a co-stimulation leads(185). Moreover, short peptides do not activate CD4 helper T cells, which are necessary for full activation of CTLs(186).

The critical point and first essential requirement in peptide-based vaccines is choosing the peptide sequence, identifying epitopes that bind to classic and non-classic HLA molecules and to determine the optimal size of peptides(187).

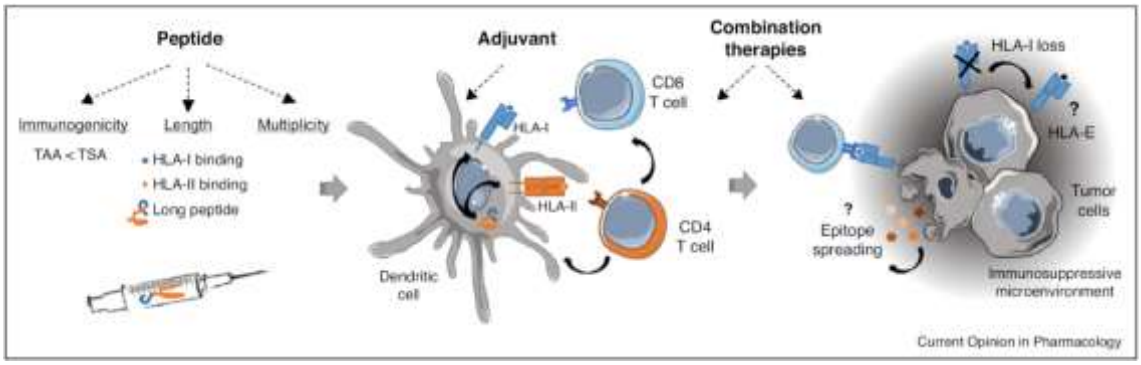


Figure 5 Peptide-based cancer vaccines. Composition of the vaccine and mechanism of action. (Reproduced with the permission from Ref (187), copyright 2019, Elsevier Ltd on behalf of Acta Materialia Inc.)

To overcome the low immunostimulation, adjuvants have been employed in the formulation of peptide-based vaccines(186). Currently, a restricted list of adjuvants has been mostly used, including Montanide ISA-51 (IFA), TLR agonists, and GM-CSF(188). However, these adjuvants display limited potency(189). In combination with the immunosuppressive TME, they are now considered to be major impediments to vaccine efficacy(190). In an effort to improve the efficacy of such vaccine, nowadays conjugate therapies are tested(191).

Various viruses have been used in the formulation of virus-based vaccines. Due to their ability to stimulate the immune system by both innate and adaptive mechanisms, they promote a strong and durable response(192). The most common viral vectors used for vaccine formulation are poxviruses, adenoviruses, herpesviruses, orthomyxoviruses, picornaviruses, paramyxoviruses, reoviruses, rhabdoviruses, and alphaviruses(192). Replication-defective or attenuated versions are preferred for safety(192, 193). However, like other vaccines, they also have disadvantages. Our antiviral immune response neutralizes the vector, consequently limiting repeat vaccination(194). To overcome this issue often a prime-boost strategy is employed which consists in delivering firstly the

antigen by one virus vector followed by a boost with the same tumor antigen delivered by another viral vector or vector type(195).

DNA- and RNA-based vaccines present several advantages(196). The first of these is their easy manufacturing, decreasing the costs and allowing the distribution also in poorer countries(197). Furthermore, they can highly stimulate DCs through more than one pathway(198). Consequently, they promote a specific immune response with a good safety profile(156). In addition, DNA and RNA-based vaccines allow repeated vaccination and are not restricted by the patient's HLA type(199). RNA cancer vaccines may offer advantages over DNA vaccines(200). The mRNA sequences for RNA-based vaccine are easier to manufacture *in vitro* conditions(201). Moreover, the sequence does not need to be integrated into the genome, so their oncogenic potential is minimal(199). To overcome obstacles, such as low DNA / RAN uptake by DCs and tumour-suppressive environment, combined strategy therapies are currently employed(202-204).

In each case, these vaccines are designed to stimulate, activate, maturate, and proliferate B or T lymphocytes by presenting tumor antigen complexed with MHC molecules to them(205). All these strategies have advantages and disadvantages being still in the process to develop. The summary of the advantages and disadvantages of each vaccine strategy is presented in *Table 1*.

Table 1 Summary of advantages and disadvantages of vaccine strategy.

	Cellular vaccines	**Peptide vaccines**	**Viral vector vaccines**	**DNA/RNA vaccines**
Advantages	- High immunogenicity - Control of antigen presentation	- Low toxicity - Easy to produce	- High immunogenicity - Easy to produce	- Easy delivery of multiple antigens - Induction of cellular and humoral immunity
Disadvantages	- Expensive and difficult to produce	- Low/moderate immunogenicity - Restricted to the HLA subtype - Expensive to produce	- Potential high immunogenicity - Risk of undesired infections - Immune reacting under the vector	- Low immunogenicity

2.2.2.6 Combined therapies

The approaches above suggest that soon cancer immunotherapy will consist of the combined treatments for majority patients with different cancer types, whereas monotherapies approaches will not be enough(206, 207). The employment of combined immunotherapies will allow fighting against cancer in all possible ways(208). The main purpose of effective combination strategies is to interfere with the activity of four different therapeutic nodes (*Figure 6*). These nodes include: (i) elimination of immune

suppression; (ii) inducing immunogenic tumor-cell death to promote tumour-antigen release; (iii) enhancing APC function; and (iv) enhance the effector-cell (memory T-cells and macrophages) activity by using appropriate stimulating agents(206). A clear understanding of the TME can help to determine which immunosuppressive pathways become activated that allows cancer survival(209). Consecutively, this will allow us to combine therapeutic strategies that are an asset to each patient.

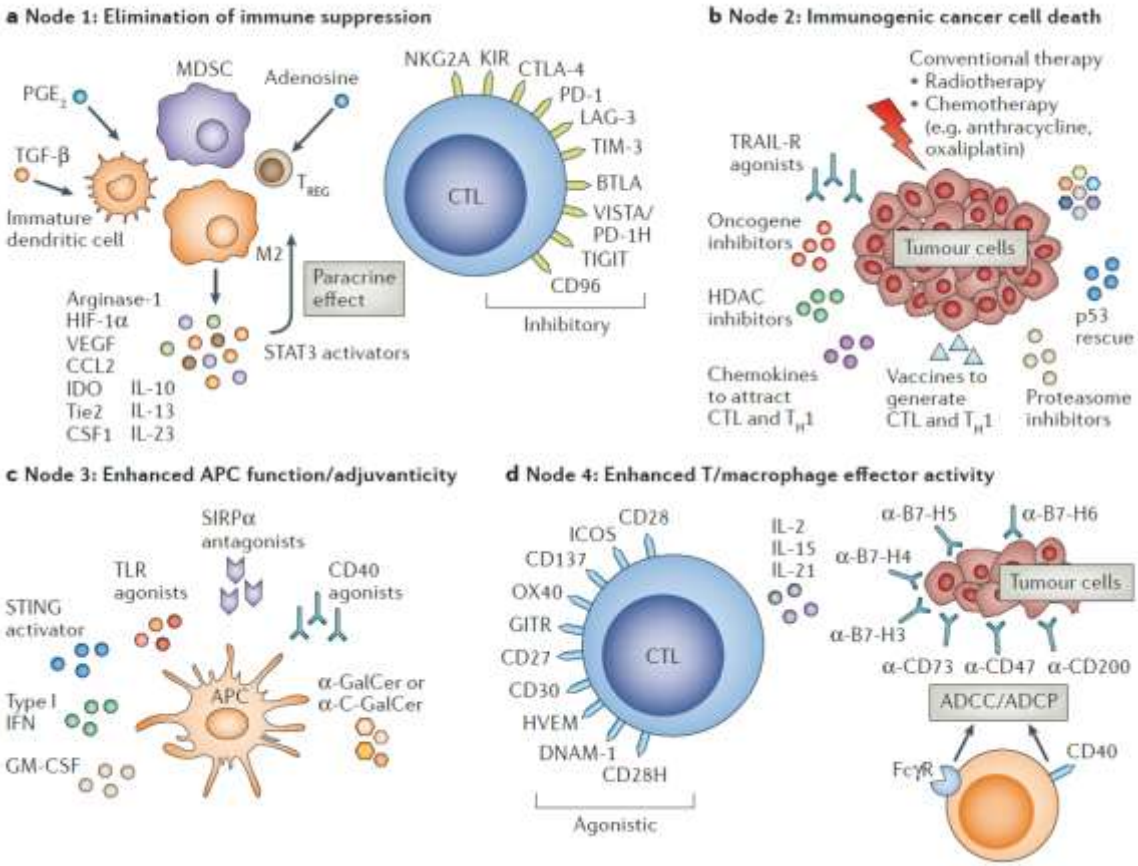


Figure 6: Four nodes to be target by cancer therapies. Different mechanisms of immunity can be modulated to enhance the efficiency of cancer immunotherapy. (Reproduced with the permission from Ref (204), copyright 2015, Macmillan Publishers Limited. All rights reserved.)

The combination of strategies can be done not only between different arms of immunotherapy, but also with other types of therapies, such as radiotherapy, chemotherapy, targeted inhibitors of different oncogenic signalling pathways and surgery(206). For example, the evolution of immunosuppressive and defensive mechanisms of cancer cells changes the TME and tumour survival(210). Thus, immune checkpoints inhibitors (CPIs), such as anti-PD-1 and CTLA-4, will probably form the base of many future cancer treatments, allowing their combination with other strategies(211, 212).

New strategies are discovered to improve cancer immunotherapy. One of them uses nanomaterials (*Figure 7*)(213). In many cases, nanomaterials are even required for promoting effective vaccine delivery(214). Indeed, the past 30 years have demonstrated the great success of various nanomaterials employed in cancer diagnosis and therapy(215).

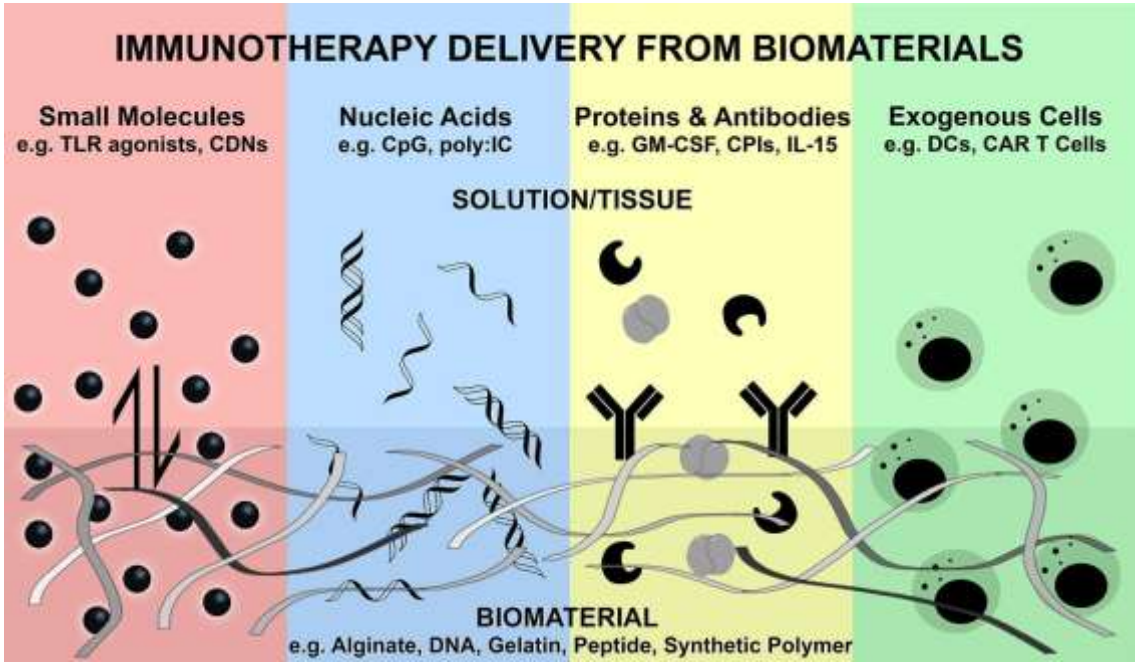


Figure 7 Combination of various nanobiomaterials-based vaccines to overcome issues of delivery process, conservation conditions and/or transportation procedure, etc. (Reproduced with the permission from Ref (216), copyright 2019, Elsevier Ltd. All rights reserved.)

Nanoparticle based on biomaterials strategies in combination with immunotherapy may help to overcome the current therapeutic obstacles and unravel a new paradigm in the treatment of cancer(217).

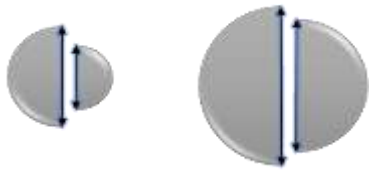
3. Nanomaterials

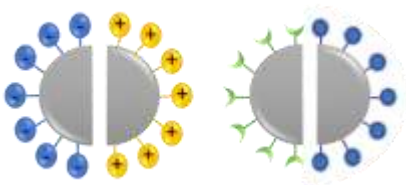
Nanomedicine is based on structures at the nanometre scale(218). These structures can be composed of different nanomaterials, presenting different size and shapes. The surface of these nanostructures can be functionalized, providing a mimetic property to biological macromolecules(219). The characterization of these nanostructures is fundamental because their properties, such as optical, electronic, and magnetic, depend directly on their size, shape, and surface (*Table 2*)(220). Depending on the synthetic method the final characteristics of the NPs will vary, so in this case, the synthesis method is a critical point to manufacture the desired NPs(221). The physicochemical properties of NPs comprehensively determine their interactions with physiological systems(222).

Table 2 Characterization of different types of nanoparticles

<table>
<tr><td>Size and Shape

The size and shape of NPs plays an important role in cellular uptake. Several studies suggest that the optimum size for NPs to attain the highest cellular uptake in certain cells, such as macrophages, is around 50 nm of diameter(223, 224).

Moreover, other studies, maintaining the size constant and changing only the shape, have demonstrated that rod-shaped NPs undergo lower cellular uptake than spherical NPs(224, 225).</td><td> 
  </td></tr>
<tr><td>Ligands and Charge

Electrostatic interactions between charged NPs and the cell membrane are very important(226). Several studies employed computer dynamics simulation to study the interaction of CM with charged NPs. The interaction between cationic NPs and phospholipid membranes showed a more favourable thermo-dynamical interaction than their uncharged NPs, which allow them to trigger off the membrane-wrapping phenomena(227). Comparing between</td><td>  </td></tr>
</table>

cationic, hydrophobic, and anionic NPs, positively charged NPs demonstrated a stronger disruptive influence on the bilayers. In addition to the charge, the surface of the NPS can be functionalized with ligands. They may be, for example, antibodies or other proteins that impart selective properties to target specific cells(228).	
Coating There is a wide variety of nanomaterials used to manufacture NPs. Not all of these materials exhibit favourable characteristics for good stability, specificity or low toxicity. To overcome this issue, coating techniques are employed to provide advantageous characteristics to the NPs(229). The coating may confer properties, such as hydrophobicity/liposolubility, binding bands, and couple charges (negative or possible).	

The physicochemical characteristics of NPs are not conferred solely by their nanostructure, the type of nanomaterial used for their production is equally important(230). Nanomaterials can be classified into organic and inorganic particles. The organic NPs include: (i) polymeric NPs (231), (ii) micelles (232), (iii) liposomes, (iv) dendrimers(233), (v) apoferritin(234) and (vi) viral NPs(235). As for the inorganic NPs, they include (i) metal NPs(236) such as gold NPs(AuNPs)(237), iron oxide NPs (superparamagnetic iron oxide NPs (SPIONs))(238), silver NPs (AgNPs)(239), (ii) quantum dots (QD) (240), (iii) carbon nanotubes (CNTs) (241), (iv) porous silicon and silica NPs (PSNP)(242), (v) porous silica NPs(243) and (vi) hybrid nanogels (organic-inorganic) (244).

In addition to characterization, a big effort is necessary to improve and generalize NPs' production, purification and separation methods(220, 245). In parallel to that, it is vital to carefully assess the behaviour of NPs inside the human body to design optimal formulations(246).

4. Nanoparticle for Cancer Immunotherapy: Nanovaccines

In the vaccine complex, each component has its purpose(247). The fundamental components are antigens that confer the specific immune stimulation, adjuvants which enhance the potency of stimulation and carriers that transport and deliver the antigens. Nanotechnology is usually employed as a carrier in cancer immunotherapy vaccines (248). In the mode of action of vaccines, nanocarriers are responsible for delivering the antigen in specific time and space, increasing the efficacy of the vaccine(249). The nanocarriers employed as carriers can be divided into three classes: biogenic, synthetic and semisynthetic (*Figure 8*)(250).

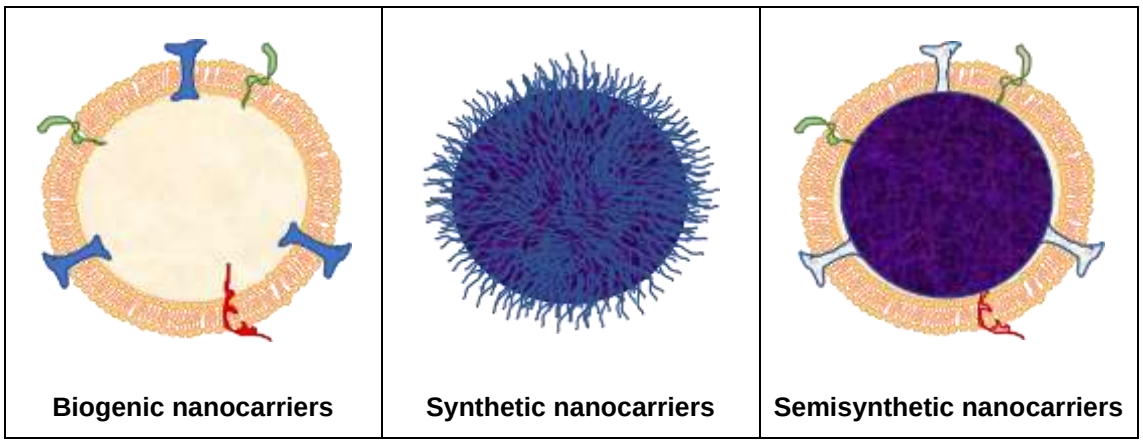


Figure 8 Schematic representation of different types of nanocarriers used for formulation of nanovaccines.

Biogenic nanocarriers are nanostructures derived from biological materials. Characteristics of those nanocarriers include great biocompatibility and biodegradability, as well as low toxicity. The representatives of this class are the exosomes (structures secreted by various cells like T cells, B cells, cancer cells, and APCs)(251) and the outer membrane vesicles (OMVs) (structures formed from the outer membranes, such as Gram-negative bacteria's membrane)(252). Nevertheless, exosome nanostructures retain big challenges related to their cost and manufacturing time at large scales(253). Bacteria-derived OMVs promote an increasing immunostimulatory signal compared with the exosomes due to their high immunogenicity(254).

Purely synthetic nanocarriers are usually directly bonded to the antigens, which are subsequently delivered to the target site or can be loaded within the nanocarrier(250). A bond between the antigen and NPs is possible when the NPs' surface has functional groups, or through an intermediate (255).

Semisynthetic nanocarriers are composed of the biogenic and synthetic materials at the same time. This class of nanocarrier combines the advantages of the other two classes. On one hand, they present good biocompatibility and low toxicity that is particular of biogenic nanocarriers. On the another hand, they are relatively easy to be reproducibly manufactured at large-scale, which is a synthetic nanocarrier attribute(250). The possible conjugations of biological and synthetic materials can be very different.

Usually, these nanostructures are organised with the synthetic part making a core and the biogenic part making a cover to camouflage the undesirable properties of the synthetic material. The biogenic part can be cell membranes(256) - using patients' cancer cells as a source; virus-like particles (VLPs) that are non-infectious due to nonexistence of virus genetic material(257); and endogenous protein such as albumin that due to its size complex permits an efficient lymphatic draining and intracellular uptake(258, 259).

In this work, passion fruits-like NPs (pfNPs) will be investigated as semi-synthetic nanocarriers for the creation of cancer-immunotherapy vaccines.

4.1 Passion Fruits-Like Nanoparticles

In 2015, Cassano *et al.* introduced a novel type of nanostructures with a core of 3 nm gold nanoparticles fixed in a polymeric array coated by a biodegradable silica shell. This nanoparticle was renamed pfNPs due to its similarity with this fruit (*Figure 9*)(260).

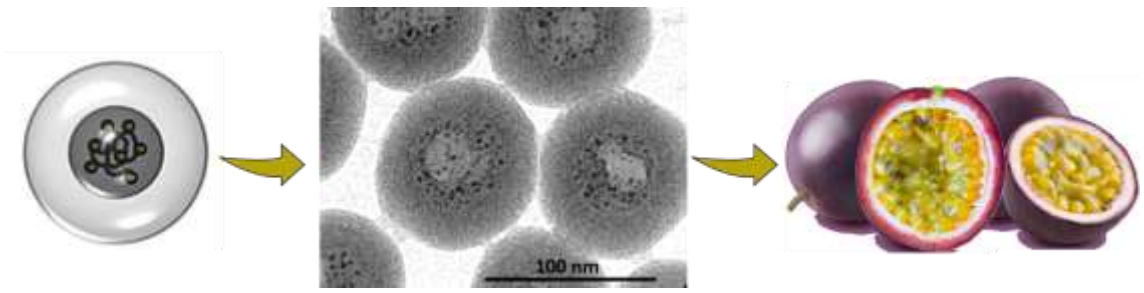


Figure 9 Schematic representation of pfNPs with the correspondent TEM imaging and their similarity with passion fruit. (Adopted with the permission from the reference (261) , copyright 2017, American Chemical Society)

Metal-based NPs have been quite widely studied showing an increasing potential for theranostics application(262). When a formulated nanotechnology-based drug arrives in clinical trials, it impacts with one of the biggest issues of nanomedicine, its excretion, to prevent its accumulation in the body. Physiologically, NPs with a diameter > 6 nm are excreted by liver and spleen, while those with a diameter < 6 nm freely eliminated by the kidneys(263). Remarkably, the average diameter of most of the metal NPs proposed for

in vivo theranostics is over 20 nm. The excretion process of those NPs is extremely slow and inefficient leading to accumulation, which consequently can cause toxicity and/or wrong diagnosis(264). On one hand, the kidney physiology requires that the nanoparticles should be < 6 nm size to hold good excretion, and on the other hand, the medicine requires that they should be > 20 nm to better exhibit their theranostic effects.

Cassano *et al.*, whit their novel nanostructures, could combine the optical behaviour of metal NPs (theranostic effects) whit a potentially complete clearance, making of them a potential solution for bioaccumulation issues in nanomedicine(261).

4.1.1 Nanoparticles Fabrication

The general synthetic protocol introduced by Cassano *et al.* can be summarized in three steps (*Figure 10*). Firstly, gold NPs were coated by (-) poly(sodium 4-styrene sulfonate) (PSS). Then, they were aggregated in spherical arrays by the (+) poly(D- or L-lysine) (PL) through ionic interactions. Finally, the silica shell was grown around the formed core by a modified Stöber method(265). The detailed synthetic protocol can be found in reference(266).

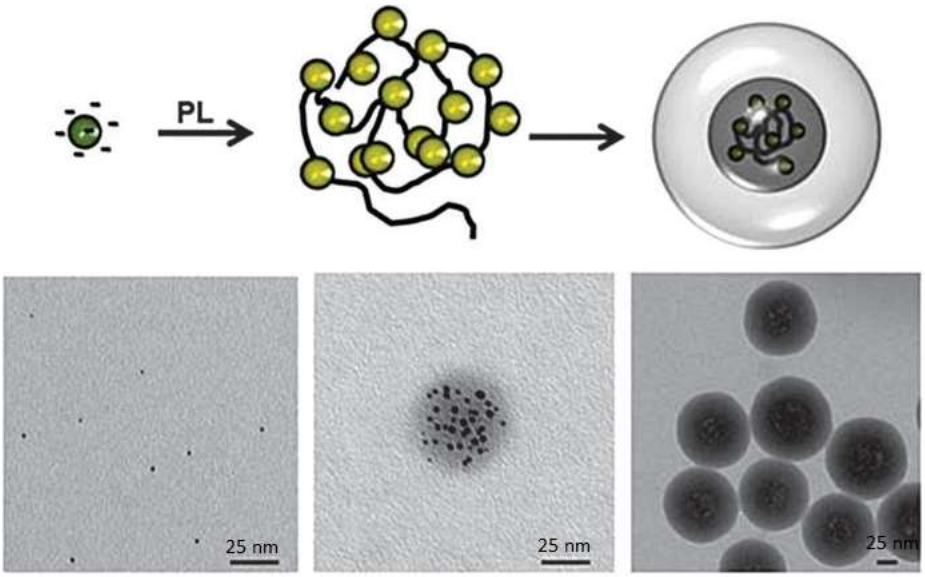


Figure 10 Representative scheme for the general formation of the pfNPs with the respective TEM imaging of each step. Negative gold NPs are assembled in spherical arrays of poly(L-lysine). These cores are then coating with silica shells. (Adapted with the permission from the reference (260), copyright 2015, The Royal Society of Chemistry)

To facilitate the execution of certain experiments pfNPs were also modified with fluorochromes, such as AlexFluor-647 (*Figure 11*). The fabrication process differs only in one step, before starting, the (+) poly(D- or L-lysine) (PL) is covalently functionalized with the fluorochrome. Detailed synthetic protocol can be found in reference(267).

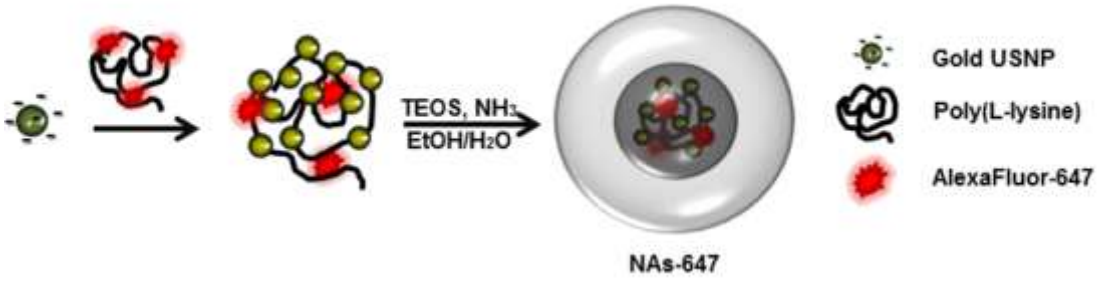


Figure 11 Representative scheme for the general formation of the pfNPs with AlexaFluor-647. (Adapted with the permission from the reference (267), copyright 2018 American Chemical Society under an ACS AuthorChoice License).

4.1.2 Biodegradability and Excretion pathways

In 2015, when Cassani *et al.* introduced those silica-gold nanostructures, they mentioned that its silica shell degrades in full serum in a few hours(260). This phenomenon was the main reason for claiming that these NPs could promote an efficient renal clearance of the silica shell-capsule and its content. Later, the same group demonstrated the NPs' degradation in 48h by TEM images. The degradation happened inside the endosomes of MIA PaCa-2 cells. The images show the internalization of NPs at t = 0, then an early stage of degradation in the endosomal compartment at t = 24 h and finally the complete degradation at t = 48 h (*Figure 12*)(268).

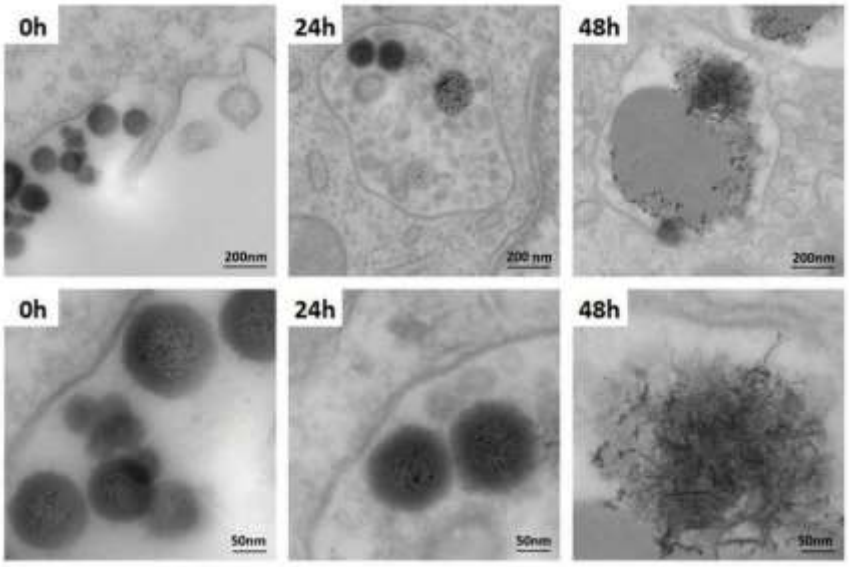


Figure 12 TEM images proving the biodegradation of pfNPs after 48h in MIA PaCa-2 cells. (Adapted with the permission from the reference (268), copyright 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim).

The degradation products of these nanostructures are polymers, ultrasmall gold nanoparticles (USAuNPs) and silicic acid(260, 261, 268). Due to their physical-chemical

properties, the authors have been suggested a potential renal clearance for the building blocks. Later, in 2018, an *in vivo* assay was performed where they proved the renal elimination as a major elimination pathway. Moreover, they find the USGNPs in faeces confirming the existence of biliary elimination pathways(269).

4.1.3 Biocompatibility and Toxicity

To assess the developmental toxicity of pfNPs, d'Amora *et al.* have employed zebrafish (*Danio Rerio*) for *in vivo* assay(270). The study was performed by exposing the zebrafish to different pfNPs concentration and subsequently assessing the resultant toxicity at different stages of development. The pfNPs were internalized and detected in specific tissues, which prove that they can pass through the chorion of the embryo. The results were extremely good, according to the OECD guidelines(271): these nanostructures presented no significant toxic effects for zebrafish. Moreover, the results showed that the pfNPs do not induce mortality, as well as any developmental defects, or alterations on the hatching rate and behaviour of zebrafish. The pfNPs were considered as a striking biocompatible nanostructure in zebrafish(270).

In another *in vivo* assay, the toxic effects of pfNPs were evaluated in healthy murine models(269). To investigate a possible toxicity in the excretion organs, liver and renal histologies were analysed (timepoints = 2, 6, and 10 days) after single injection through the tail vein of pfNPs (4.5 mg/200 µL, 1 mg/mL in gold). The results have confirmed the absence of hepatotoxicity and nephrotoxicity. The histological sections at the three-time points show a normal cellular architecture without any evidence of inflammation or other pathological signs(269).

These data increasingly reinforce the strong biocompatibility of these novel nanostructures.

5. Aims of the Study

The main aim of the present study was to investigate the *in vitro* immunological profile of pfNPs in human and murine cell lines.

In particular, the detailed objectives of this work were:

- To evaluate the cytocompatibility of the NPs in murine (RAW 264.7) and human (KG-1, and BDCM) immune cells, using the intracellular ATP activity as an indicator of cell viability at different time points (6h, 24h, and 48h), and to employ the Varioskan Flash reader to measure the luminescence of CellTiter-Glo® reagent degradation products by the presence of ATP.
- To quantify their uptake in murine (RAW 264.7) and human (KG-1) immune cells, by employing flow cytometry (FCM) and transmission electron microscopy (TEM), and to use the fluorescent pfNPs-647 at 5 µg/mL for two time points (3h and 6h).
- To analyse their immunostimulative potential in murine cells (RAW 264.7) by using FCM (flow cytometry) and to analyse the maturation markers (CD80/CD86) on the cell's surface incubated at different concentrations of NPs (5 µg/mL and 100 µg/mL) for 24, 48 and 72h.

Experimental Section

6. Materials and Methods

The NPs used in the *in vitro* studies were kindly produced by Domenico Cassano, PhD student from the research group of Center for Nanotechnology Innovation NEST, Italian Institute of Technology, P.zza San Silvestro, 12 - 56126, Pisa, Italy. Two types of NP's were used, the standard one prepared with final concentration 4000 µg/mL and the fluorescent ones NPs-647 whit final particle concentration 2000 µg/mL. The general synthesis method of pfNPs can be found in reference(266).

6.1 Passion Fruits-Like NPs Characterization

After production, the nanoparticles were also characterized in NEST, Italian Institute of Technology, Pisa, Italy. Their diameter and zeta (ζ)-potential were assessed, as well as its morphology in TEM.

6.1.1 Size and ζ-Potential

All the measurements were obtained using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd, UK). For the hydrodynamic diameter and polydispersity index (PdI) measurements we used a disposable polystyrene cuvette (SARSTEDT AG & Co., Germany) while the ζ-potential was measured in a disposable folded capillary cell (DTS1070, Malvern, UK). The sample was recovered from its original solvent (EtOH 96%) by centrifuging the particles at 13000 rpm for 3 min, discarding the supernatant and resuspending in Milli-Q-water. For each measure an aliquot of 25 µg of recovered standard NP's was dispersed in 1 mL of Milli-Q-water.

6.1.2 NPs' Morphology

The NPs' morphology was evaluated by transmission electron microscopy (Jeol JEM-1400, Jeol Ltd, Japan). The samples were prepared by dropping 10 µL of the solution of NPs (4000 µg/mL) on the bottom of a sheet of parafilm (Parafilm "M" PM-996, LABORATORY FILM, Bemis, Finland). Then the carbon-coated copper grids (FUF 200-CU Mesh Copper; Electron Microscopy Sciences, USA) were posed on top of the droplet on the surface of the parafilm, so that it coalesces with the droplet's surface and then reversed, retaining the sample in the grid. Then the grids were left to dry at room temperature overnight.

6.2. Stability Studies

To evaluate the effect of various solvents and buffers on the standard pfNPs, the hydrodynamic diameter, PdI and ζ-potential in Milli-Q-water were considered as the reference point. NP's stability was studied with tree different solvents, in Dulbecco's Modified Eagle's Medium (DMEM) (DMEM-High Glucose, HyClone®, USA) with 10 % (%v/v) HIFBS (HIFBS, Gibco®, by Life Thecnology™, ThermoFisher, USA)), in 1× PBS buffer (Phosphate buffered saline, HyClone®, USA), and in Human Fresh Frozen Plasma 24 (FFP24): PBS-Ethylenediamine Tetraacetic Acid (EDTA)1:1 (Finnish Red Cross). The plasma was melted at the room temperature before use and then centrifuged at 4000 rpm for 5 min and filtered with a 0,2 µm membrane filter (Acrodisc®, 32 mm Syringe Filters with 0.2 µm Supor® Membrane, Non-Pyrogenic, UK) using a 50 mL syringe (50 mL (60mL) Syringe, Soft-Ject®, Italy). For each study 200 µg of recovered NPs were dispersed in 1.5 mL of solvent (10% DMEM, PBS or FFP) in a 3 mL glass vial. For the duration of the experiment, the samples were mixed on a stirring plate (2mag, magnetic ^{e} motion, MIX 15 eco, Sigma-Aldrich, Finland). At each time point an aliquot of 200 µL was taken and re-suspended in 800 µL of Milli-Q-water inside the polystyrene cuvette (SARSTEDT AG & Co., Germany) for measuring the size, followed by ζ-potential measure of the same sample using a folded capillary cell (DTS1070, Malvern, UK) on Zetasizer Nano ZS (Malvern Instruments Ltd, UK). The time points studied were 0, 5, 10, 15, 30, 60, 90 and 120 min at room temperature.

6.3 *In Vitro* Studies

6.3.1 Cell Lines and Culture Conditions

The specification of each type of the cell lines and culturing conditions is described in detail below.

6.3.1.1 RAW-264.7

An immortalized macrophage derived from the mouse. Biosafety level 2 (RAW 264.7, ATCC®, TIB-71™). Cells were stored at the temperature −196 °C in liquid nitrogen and were used at passages #11−25. The cells were cultured in medium composed by DMEM (DMEM, High Glucose, HyClone®, USA) supplemented with 10% Heat Inactivated Fetal Bovine Serum (HIFBS, Gibco®, ThermoFisher, USA), 1% non-essential amino acids (NEAA, Gibco®, ThermoFisher, USA), 1% (200 mM) L-glutamine (Gibco®, ThermoFisher, USA) and 1% (100×) Penicillin and Streptomycin (PEST; Gibco®, ThermoFisher, USA). The cells were maintained at 37 °C in an atmosphere of 5% CO_2

and 95% relative humidity in a standard incubator (16 BB gas, Heraeus Instruments GmbH, Germany). The collection of the cells prior to each experiment was performed with a 0.25% Trypsin solution (0.25%Trypsin (10×), Gibco®, ThermoFisher, USA). RAW-264.7 cells are adherent type of cells. To avoid cell differentiation, they were passaged before reaching 100% confluence (80% optimal). The subculturing of the cells was performed starting with the removal of the medium and adding 0.25% trypsin solution (1 mL into 75 cm^2 flasks), followed by incubation for 5 min at 37 °C in the incubator. After confirming that the cells detached, the trypsin was neutralized with the addition of 9 mL of DMEM medium and the solution was transferred to a falcon. The solution containing the cells was centrifuged at 1500 rpm for 5 min, the supernatant discarded, and the cells resuspended in a fresh medium. A predetermined amount of cells was transferred to a new flask (75 cm^2) in 12 mL of medium.

6.3.1.2 KG-1

An immortalized lymphoblast derived from a man with acute myelogenous leukemia (AML). They present a biosafety level 1 (KG-1, ATCC®, CRL-8031™). Cells were stored at the temperature of −196 °C in liquid nitrogen and were used at passages # 19−21. The cells were cultured in medium composed by Iscove's Modified Dulbecco's Medium (IMDM, Gibco®, ThermoFisher, USA) supplemented with 10% HIFBS (Gibco®, ThermoFisher, USA), 1% (100×) NEAA (Gibco®, ThermoFisher, USA), 1% (200 mM) L-glutamine (Gibco®, ThermoFisher, USA) and 1% (100×) PEST (Gibco®, ThermoFisher, USA). The cells were maintained at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity by a standard incubator (16 BB gas, Heraeus Instruments GmbH, Germany). KG-1 cells are non-adherent cells growing in suspension. Cultures was maintained by the addiction of fresh medium every 2 to 3 days depending on cell density (ideal growing concentration 2×10^5 cells/mL). When the culture medium in the flask needed to be changed completely, the medium containing the cells was centrifuged at 1100 rpm for 5 min, followed by discarding the supernatant and resuspending in a predetermined amount of fresh medium.

6.3.1.3 BDCM

BDCM are B cell line (lymphoblast) derived from a man with M5a Leukemia. They present a biosafety level 2 (BDCM, ATCC®, CRL-2740™). Cells were stored at the temperature −196 °C in liquid nitrogen and were used at passages # 9−14. The cells were cultured in medium composed by Roswell Park Memorial Institute Medium (RPMI 1640, HyClone®, USA) supplemented with 10% HIFBS (Gibco®, ThermoFisher, USA), 1% (100×) NEAA (Gibco®, ThermoFisher, USA), 1% (200 mM) L-glutamine (Gibco®,

ThermoFisher, USA) and 1% (100×) PEST (Gibco®, ThermoFisher, USA). The cells were maintained at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity in a standard incubator (16 BB gas, Heraeus Instruments GmbH, Germany). BDCM cells are non-adherent cells growing in suspension. Cultures was maintained by the addiction of fresh medium every 2 to 3 days depending on cell density (ideal growing concentration 3×10^5 cells/mL). When the culturing medium in the flask need to be substituted completely, the medium containing the cells was centrifuged at 1100 rpm for 5 min, the supernatant was then discarded and the pellet of cells was resuspended and a predetermined amount of cells was added to fresh medium in a new flask.

6.3.2 Cell viability Studies

To determine the toxicity of pfNPs onto RAW 264.7, KG-1, and BDCM cells, the intracellular ATP activity was measured at different time points. The protocol for the RAW 264.7 (adherent cells) was different than for KG-1 and BDCM (suspension cells).

RAW 264.7: About 1×10^4 cells suspended in culture medium (100 µL) were seeded in each well in a 96-well plate (Corning Incorporated, USA) and allowed to attach overnight. Then, the medium was removed, and 100 µL of solution containing NPs at the different concentrations was added into each well. The plates were incubated at 37 ºC (5% CO_2 and 95% RH) for 6, 24, and 48 h. The sample solution at the different concentrations (0.5, 5, 10, 50 and 100 µg/mL) were prepared using cell medium as dispersing medium, Complete DMEM and Triton® X-100 (Fluka™, Finland) were used as negative and positive controls, respectively. All the samples/controls were pipetted in quadruplicate. At each time point, the medium was removed from the wells and the plates were washed twice with Hank's Balanced Salt Solution (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) HBSS–HEPES (pH 7.4). Then, 100 µL of HBSS−HEPES 50:50 CellTiter-Glo® reagent (Promega Corporation, USA) solution was added to each well. After mixing with an orbital shaker for 2 min to induce cell lysis, the plates were left for 15 min at room temperature and then the luminescence was measured with a Varioskan Flash reader (Thermo Fisher, Scientific Inc., USA).

KG-1 and BDCM: About 1×10^4 cells suspension in their own culture medium (50 µL) were added to each well in a 96-well plate (Corning Incorporated, USA). Then, 50 µL of solution containing NPs at the different concentrations was added into each well, and the plates were incubated at 37 ºC (5% CO_2 and 95% RH) for 6, 24, and 48h. The sample solution at the different concentrations (0.5, 5, 10, 50 and 100 µg/mL) were prepared, taking into account the dilution factor 2 (Cells suspension 50:50 NP suspension), using the cell own culture medium. Complete respective culturing medium

(10% HIFBS-IMDM for KG-1 and 10% HIFBS-RPMI for BDCM) and Triton® X-100 (Fluka™, Finland) were used as negative and positive controls, respectively. All the samples/controls were pipetted in quadruplicate. At each time point, the plates were removed from the incubator and leaved at the room temperature for 20 min. Afterwards, 100 µL of CellTiter-Glo® reagent (Promega Corporation, USA) were added directly to each well. The plates were mixed with an orbital shaker for 2 min to induce cell lysis, and then left for 15 min at room temperature. Finally, the measures of the luminescence were performed with a Varioskan Flash reader (Thermo Fisher, Scientific Inc., USA).

6.3.3 Uptake Studies

To evaluate whether the NPs are taken up by the cells, flow cytometry and TEM were employed. In this assay, we used a fluorescent pfNPs-647 in order to measure them in the flow cytometer instrument.

6.3.3.1 Evaluation of the Uptake by Flow Cytometry

RAW 264.7: About 1 mL of 5×10^5 cells/mL in 10% of HIFBS-DMEM was seeded in each well in a 12-well plate (Corning Incorporated, USA) and allowed to attach overnight. Next day, the medium was removed, and 1 mL of solution (10% HIFBS-DMEM) containing the fluorescent NPs-647 at the concentrations 5 µg/mL was added into the wells corresponding to the samples for 3 h and 6 h. 10% HIFBS-DMEM culture medium was used as a negative control. All the samples/controls were pipetted in triplicate. The plates were incubated at 37 ºC (5% CO_2 and 95% RH) until the end of each time point. Then the medium was removed and washed once with 1 mL of PBS 1× (HyClone®, USA). After that, the plate was incubated at 37 ºC (5% CO_2 and 95% RH) for 5 min with 500 µL of Versene 1× (Versene 1:5000, Gibco®, Life Technologies, USA) in each well to detach the cells. Afterwards, the Versene solution containing the cells was collected into the BD FACS tubes (BD Biosciences, USA) and centrifuged at 1500 rpm for 5 min. The supernatant was discarded, and the cells were washed three times with 500 µL PBS (1×) buffer.

KG-1: About 500 µL of 5×10^5 cells/mL in respective full culturing medium (10% HIFBS-IMDM) was seeded in a 12-well plate (Corning Incorporated, USA). Then, in the wells corresponding to the samples for 3 h and 6 h, we added 500 µL of fluorescent NPs-647 at the concentrations 10 µg/mL, so that final concentration in the wells was 5 µg/mL. Full culture medium was used as a negative control. All the samples/controls were pipetted in triplicate. The plates were incubated at 37 ºC (5% CO_2 and 95% RH) until the end of each time point. Afterwards, medium solution with the cells were collected into the BD FACS tubes (BD Biosciences, USA) and centrifuged at 1100 rpm for 5 min. The

supernatant was discarded, and the cells were washed three times with 500 µL PBS (1×) buffer.

Thereafter, all the samples (RAW 264.7 and KG-1) were read on FCM (flow cytometer) (BD Accurí equipped with a c6 autosampler, BD Biosciences, USA) two times. The first time the samples were read in PBS solution, obtaining so called "associated" results, and the second time the samples were read adding 500 µL of 0.05% Trypan Blue in PBS (Trypan Blue Stain (0,4%), Gibco®, USA) solution to each sample to quench the fluorescence emitted by particles not uptaken by the cells, obtaining so called "internalized" results.

6.3.3.2 TEM Imaging of Uptaken NPs

The TEM technique was used to investigate the intracellular uptake independently from any fluorochrome in RAW 264.7 and in KG-1 cells. To this aim, we coated glass slides (#1, VWR; USA) with poly-D-lysine (Sigma Aldrich, USA) in 12-well plates overnight. The lysine solution was then removed, and the slides were left drying. Then 3×10^5 cells were seeded in each well. The cells were left attaching overnight. Then, samples were added at the concentration of 5 µg/mL and incubated for 1, 3, or 6 h.

Upon each time point, the medium was removed, and the cells were fixed in 2% glutaraldehyde in 0.1 M of Sodium Cacodylate (NaCaco) for 30 min at room temperature. The wells were then washed twice with 0.1 M of NaCaco. Then the samples were given to the Electronic Microscopy Unit for further processing. The samples were embedded into Epox resin and cut with a cryotome. The grids were then imaged with a TEM microscope (Jeol JM 1400, Jeol, Japan).

6.3.4 Immunostimulation Studies

Flow cytometry (FCM) was used in order to monitor the immune response stimulated by pfNPs in RAW 264.7 cell line. The evaluation of the immune response consisted in the analysis of the maturation markers on the cell's surface, namely the expression of CD80/CD86 receptors. In this assay, the immune response was evaluated at different concentrations, namely 5 and 100 µg/mL, at 24, 48 and 72 h. Moreover, Lipopolysaccharides (LPS) (Sigma Aldrich, USA) 100 ng/mL was used as a positive control, whereas, cell own complete culturing medium was used as a negative control.

About 1 mL of 5×10^5 RAW 264.7 cells/mL in 10% of HIFBS-DMEM was seeded in each well in a 12-well plate (Corning Incorporated, USA) and allowed to attach overnight. Next day, the medium was removed, and 1 mL of the samples/controls were added. All the samples/controls were pipetted in triplicate and incubated at 37 ºC (5%

CO_2, 95% RH). Then the cells were collected into a 10 mL Falcon tube (Falcon™, USA). Firstly, the medium was collected, then the cell were detached using 500 µL of ice-cold PBS–EDTA 0.5 mM (PBS 10×, HyClone®, USA; 5.0 M EDTA, Gibco®, Life Technologies, USA), followed by incubation for 5 min at 37 ºC (5% CO_2, 95% RH) and collected to the correspondent falcon.

The Falcons with the samples were centrifuged at 1500 rpm for 5 min. The supernatant was discarded, and the cells were resuspended in 800 µL of PBS (1×). Afterward, each sample was divided in 4 and transferring into a 96-well V Bottom Plat (96-well Cluser, V Bottom Polyrene, Cstar®, USA), 200 µL in each well. The 4th well of each sample was used as an Internal Control (IC). The plate was centrifuged, and the supernatant discarded. Each sample (except IC) was resuspended in 30 µL of Murine Fc Block:PBS (1×) 1:15 (Murine Fc Block, TrueStainFX, Biolegend, USA) and incubated at room temperature for 10 min. The samples corresponding to IC were resuspended in 80 µL of PBS. Afterwards, in the dark, 50 µL of Anti-CD80 and 1% of Anti-CD86 in PBS (Biolegend, USA) solution was added over to Murine Fc Blocker's solution and the cells were incubated in the fridge at 4 ºC for 25 min. The cells were then centrifuged at 800*g* for 5 min and washed twice with 200 µL of PBS. Finally, the cells were resuspended in 200 µL of PBS and analysed with Accurí flow cytometer.

7. Results and Discussion

7.1 Passion Fruits-Like NPs Characterization

Firstly, we extensively characterized the NPs, particularly pfNPs' size, ζ-potential and morphology. NPs' size was evaluated in Milli-Q water by Zetasizer Nano ZS instrument. The average of their hydrodynamic diameter was 196.8 nm (± 2.3 nm) with a polydispersity index (PdI) equal to 0.065 (± 0.033). These results indicate the quite high uniformity of the dispersed NPs. Their ζ-potential measurement was equal to -29.7 mV (±0.7 mV). The current guidelines classify NPs' ζ-potential values in four classes, from ±0 to 10 mV as highly unstable, from ±10 to 20 mV as relatively stable, from ±20 to 30 mV as moderately stable, and finally >±30mV as highly stable(272). According to them, pfNPs can be considered between moderately stable and highly stable in Milli-Q water.

The pfNPs' morphology was then evaluated by TEM imaging (*Figure 13*). They present a spherical shape with a distinguishable shell and a core containing the ultra-NPs.

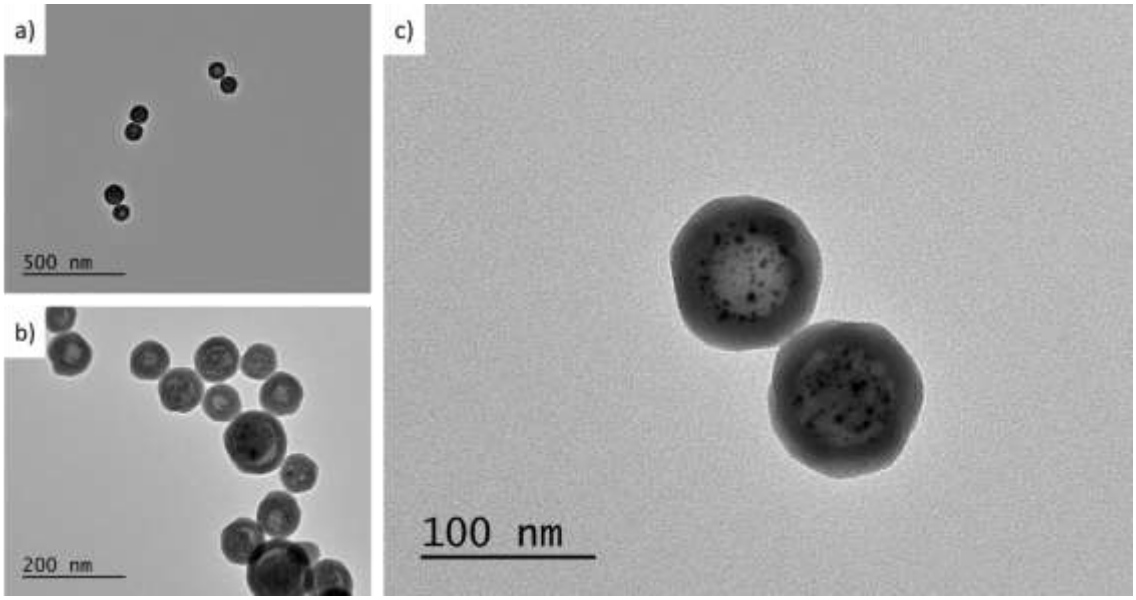


Figure 13 TEM images of the pfNPs. Scale bars are 500 nm a), 200 nm b) and 100 nm c).

7.2 Stability Studies

In the next step, the stability of pfNPs was tested in different buffers for 120 min. In 10% of FBS in DMEM medium, the pfNPs' hydrodynamic diameter remained stable, with a slight growth from 255.9 nm (±24.2 nm) (t=0) to 274.1 nm (±15.0 nm) (t=120)

(*Figure 14-a)*), while the respective ζ-potential, around −16,5 mV, was constant for 120 min (*Figure 14-d)* **o** *DMEM*). This medium was chosen as a representative to test the stability of the pfNPs in cell growth medium, to be sure that, in subsequent *in vitro* tests, the medium would not affect negatively or positively the results. In PBS (1×) buffer, despite small fluctuations in the pfNPs' hydrodynamic diameter, the NPs were stable for 120 min (*Figure 14-b*). They present 279.7 nm average of hydrodynamic size with a maximum deviation at t=15 min (315.0 nm (±13.0 nm)) and a minimum deviation at t=30 min (261.3 nm (±37.2 nm)). The pfNP's ζ-potential averaged −38.3 mV with some variations of ± 3.3 mV (*Figure 14-d)* □ *PBS*). The stability of the NPs in PBS is important as a vehicle for the eventual injection of the final formulation(273).

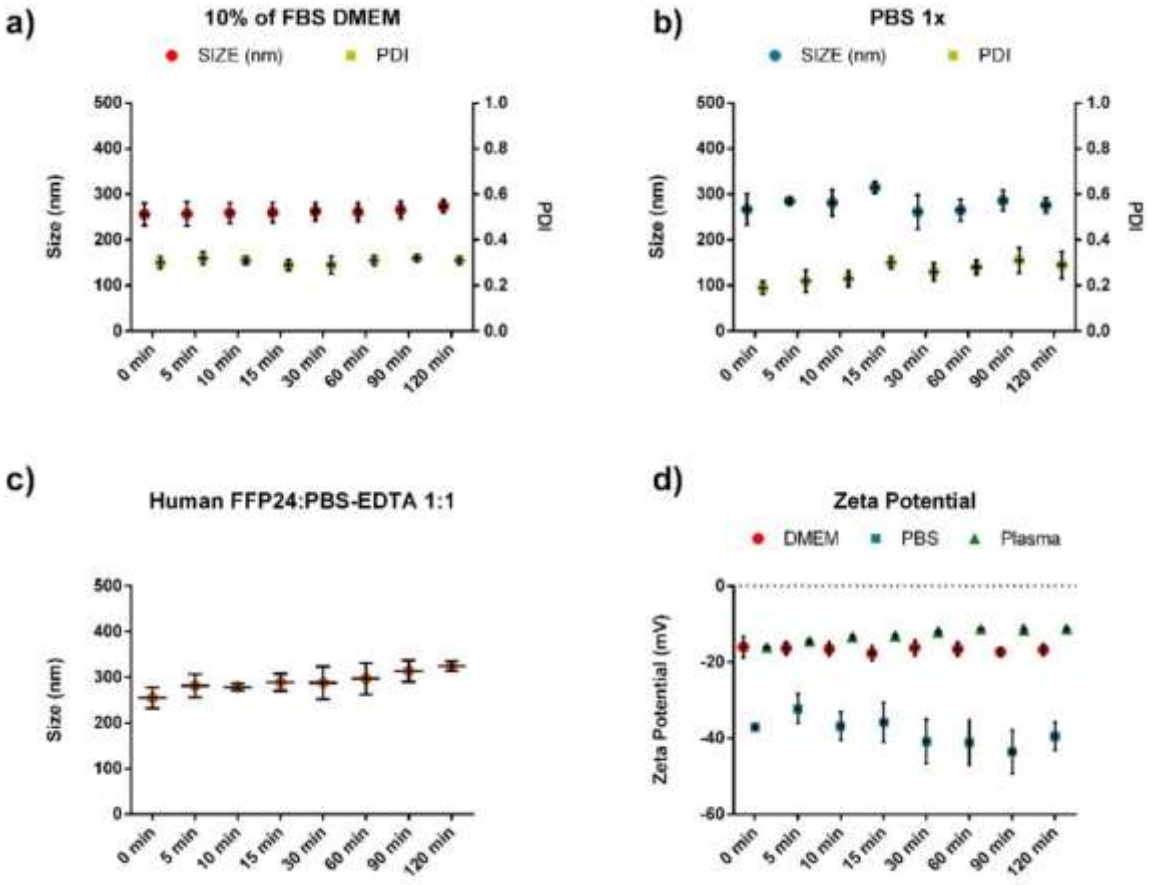


Figure 14 The pfNPs' stability relative to size for 120 min in a) 10% of FBS DMEM, b) in PBS (1×) buffer and in c) Human FFP24:PBS-EDTA 1:1. The graphic d) represent pfNPs' ζ-potential for 120 min in different buffers. Error bars reveal mean ± S.D. (n = 3).

In Human FFP24: PBS-EDTA 1:1 buffer the pfNPs were less stable compared to other buffers and their hydrodynamic size showed a slight growth from 255.2 nm (±22.9 nm) (t=0) to 325.3 nm (±10.7 nm) (t=120) (*Figure 13-c*). The respective ζ-potential showed a slight increase from −16.5 mV to −11 mV at t = 120 (*Figure 14-d)* △ *Plasma*). The study of the NPs stability in human blood/plasma is an indicative factor of biocompatibility and efficacy of therapeutics(274).

7.3 Cell viability Studies

Then, the cytocompatibility of pfNPs was assessed on three immortalized cell lines (*Figure 15*): RAW 264.7 mouse macrophages, KG 1 human macrophages and BDCM. The interaction between the NPs and immune cells is of utmost importance after administration. Moreover, the biocompatibility of the carrier directly influences the efficacy of the therapeutics(275).

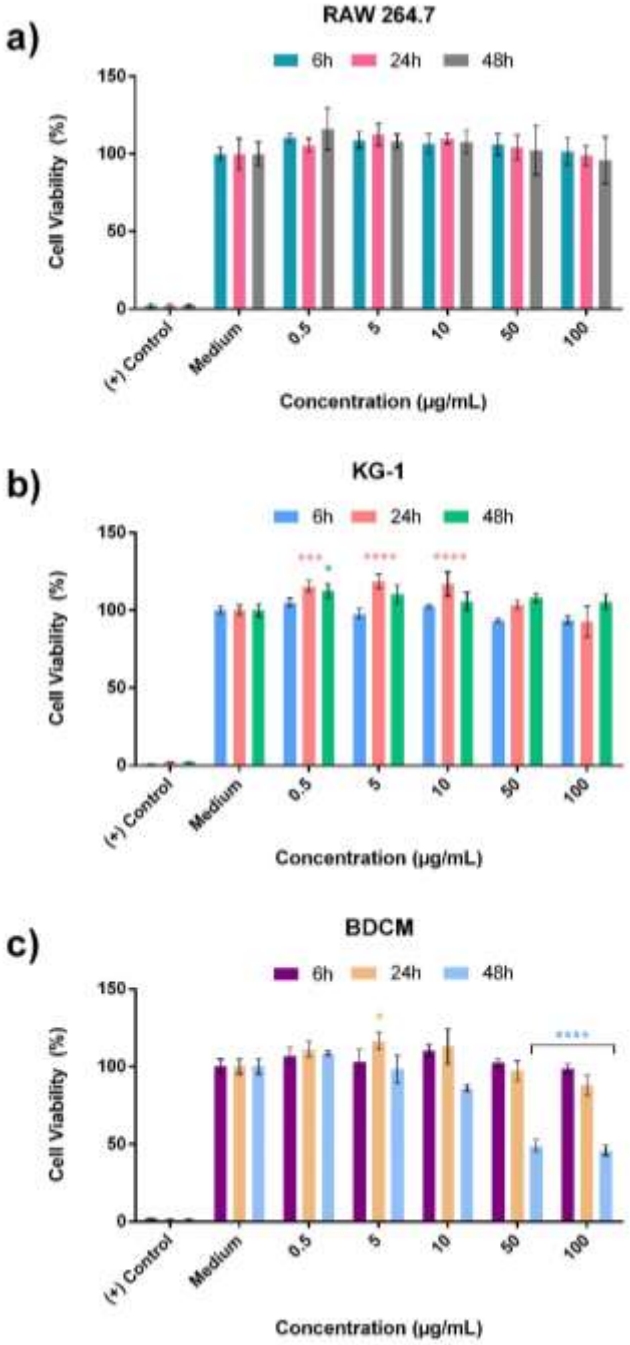


*Figure 15 Cell viability (%) of a) RAW 264.7, b) KG-1 and c) BDCM cells after incubation with the pfNPs for 6, 24, and 48 h. Complete DMEM and Triton 1% were used as negative and positive controls, respectively. The samples were analysed with two-way ANOVA statistics. The levels of significance were set by ns (not significant), *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. The significance was evaluated comparing to the negative control (complete medium). Error bars reveal mean ± S.D. (n = 3).*

In RAW 264.7 cells, after 48 h, there was no statistically significant difference between the cells incubated in the whole range of concentration assessed and the negative control (complete medium) (*Figure 15-a*). Thus, pfNPs were cytocompatible for concentrations up to 100 µg/mL. In KG-1 cells, significant higher cell viability (%) was detected to 0.5 µg/mL at 24 and 48 h (*** p <0.001 and * p <0.05, respectively), 5 µg/mL and 10 µg/mL at 24 h (****p <0.0001) concentrations compared to negative control (*Figure 15-b*). Those variations reveal an induced soft proliferative effect by pfNPs that needs to be further investigated. To date, no systematic study that could explain the proliferation phenomena observed in KG-1 is available. The rest of results showed cell viability values comparable with the negative control after 48 h. Thereby, pfNPs were considered cytocompatible for concentrations up to 100 µg/mL in these cells. In BDCM cells, a significant difference on viability values was detected at 5 µg/mL at 24 h (*p <0.05), corresponding to a soft proliferative effect and, 50 and 100 µg/mL at 48 h (****p <0.0001), corresponding to a cytotoxic effect (*Figure 15-c*). Thereby, the pfNPs were found cytocompatible for concentrations up to 10 µg/mL in these cells.

Shahbazi M. *et al*. have studied the surface chemistry effects of porous silicon NPs on biocompatibility in various cells including RAW 264.7 cells. The results showed cytocompatibility of the cells exposed to different PSi NPs (such as thermally oxidized PSi (TOPSi), thermally carbonized PSi (TCPSi), and (3-Aminopropyl) triethoxysilane functionalized thermally carbonized PSi (APSTCPSi)) up to the concentration 100 µg/mL and up to 24h(276). In another study, Fontana F. *et al*. have developed two multistage nanovaccines based on porous silica (PSi) and assess their cytocompatibility and immunostimulative properties in BDCM and KG-1 cells. They have checked three intermediate stages (thermally oxidized PSi + acetylated dextran (TOPSi@AcDEX) or spermine-modified AcDEX (TOPSi@SpAcDEX)) and two final nanovaccines (TOPSi@AcDEX + a core-shell of cancer cell membrane system (TOPSi@AcDEX@CCM) and TOPSi@SpAcDEX + a model antigen Trp2 (TOPSi@SpAcDEX-Trp2)). In general, the developed nanovaccines were cytocompatible up to the concentration of 250 µg/mL and up to 72 h in BDCM and KG-1 cells(277). These data suggest that the three cell lines evaluated are able to withstand the co-incubation with nanoparticles. The higher toxicity encountered in BDCM in the present study may be related to the smaller size of the particles(278) and the surface material (silica versus polymer) as well as the presence of gold nanoparticles within the passion fruit particles readily released within the cytoplasma at 48h(268).

7.4 Uptake Studies

The entry of many NPs into cells is considered an important step to achieve high therapeutic efficacy(279). An example are those NPs that work as a carrier that deliver the specific molecules (*i.e.*, genes, proteins, stimulating agents, drugs, and contrast agents) to the desired intracellular sites(280). As in this case, pfNPs are tested for stimulation of the immune system in order to be used further as a nanocarrier in cancer vaccines. For this purpose, the NPs first need to be uptaken by the immune cells. Therefore, the uptake of pfNPs was assessed on two immortalized cell lines: RAW 264.7 and KG-1 cells. The particles (5 µg/mL) were maintained in medium with the cells for 3h and 6h.

In the case of RAW cells (*Figure 16-a, 16-c and 16-e*), the samples at 3 h present statistically significant higher values of MFI APC (%) and % of positive cells compared with the controls only for associated samples which suggest that the uptake process is in the beginning. Thus, at 6 h statistically significant higher values (****p <0.0001) of associated and uptaken samples were observed compared with controls in both MFI APC (%) and % of positive cells (*Figure* 16-a and 16-c), respectively. This data showed an increasing uptake over the time, thus a time dependent uptake with very high cell association. Herranz-Blanco B. *et al*. have carried out an *in vitro* macrophage-mediated phagocytosis test whit PSi NPs and PSi polymeric composite (PSi−PC) using RAW 264.7 macrophage cells. The results showed a percentage of positive cells for the PSi NPs and PSi–PCs as of 93.5% and 24%, respectively after 3h incubation whit the NPs(281). These data suggest that the surface material (porous silica versus polymeric composite) of NPs greatly interferes with the cellular uptake. The percentages of uptaken PSi NPs after 3h incubation are similar to the results of pfNPs after 6h incubation with the same cells. The slower uptake of pfNPs may be related to the different size of the particles and different surfaces (porous silicon vs silica) (282).

In case of KG-1 cells, two populations of cells were detected in flow cytometry. Both populations showed little to no uptake, as seen from the MFI APC (%) and % of positive cells values compering with the respective controls (*Figure* 16-b, 16-d and 16-f).

To date, no study evaluating the uptake of silica-based NPs or other NPs using KG-1 cells is available to compare with the results of this study.

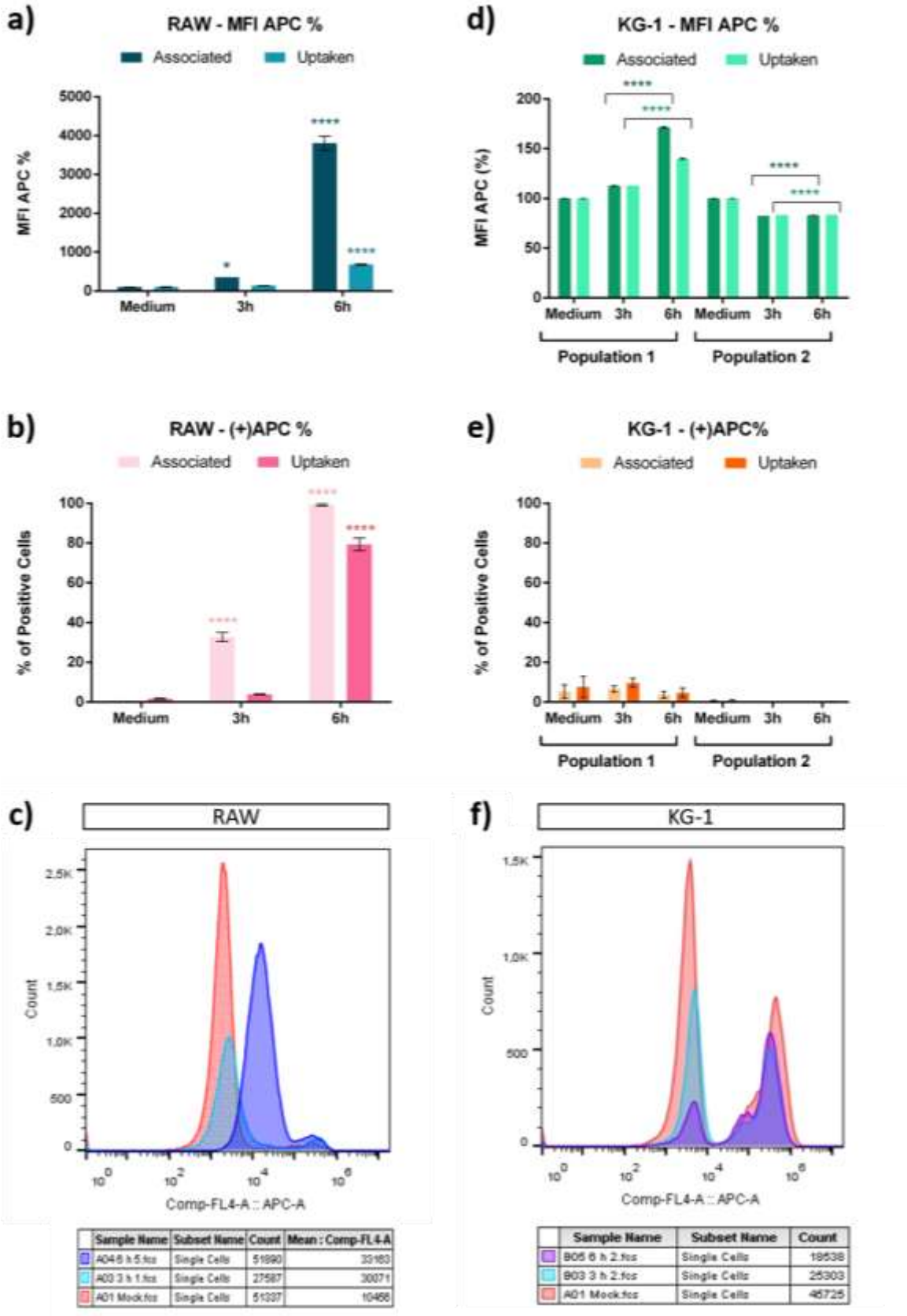


*Figure 16 MFI APC (%) (a) and d), and % of positive cells b) and e)) values of the RAW 264.7 and KG-1 cells after incubation with the fluorescent pfNPs-647 (5 µg/mL) for 3 and 6h. Complete DMEM was used as negative control. The samples were analysed with two-way ANOVA statistics. The significance was evaluated comparing to the negative control (complete medium). The levels of significance were set by *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 probabilities. Error bars represent mean ± S.D. (n = 3). Figures c) and f) represent examples of histograms of pfNPs' fluorescence in the samples RAW 264.7 and KG-1 cells incubated for 3 and 6h with the pfNPs (5 µg/mL).*

TEM pictures of RAW 264.7 cells captured after 1, 3, and 6 h of incubation with pfNPs-647 (5 µg/ml) showed the uptaken of NPs. Between 1 h (*Figure 17-a–d*) and 3 h (*Figure 17-e–h*) were detected membrane-associated pfNPs-647, in the process to be uptaken and already internalized NPs. After 6 h, all captured images were of pfNPs internalized in the endocytic vacuole (*Figure 17-i–l*).

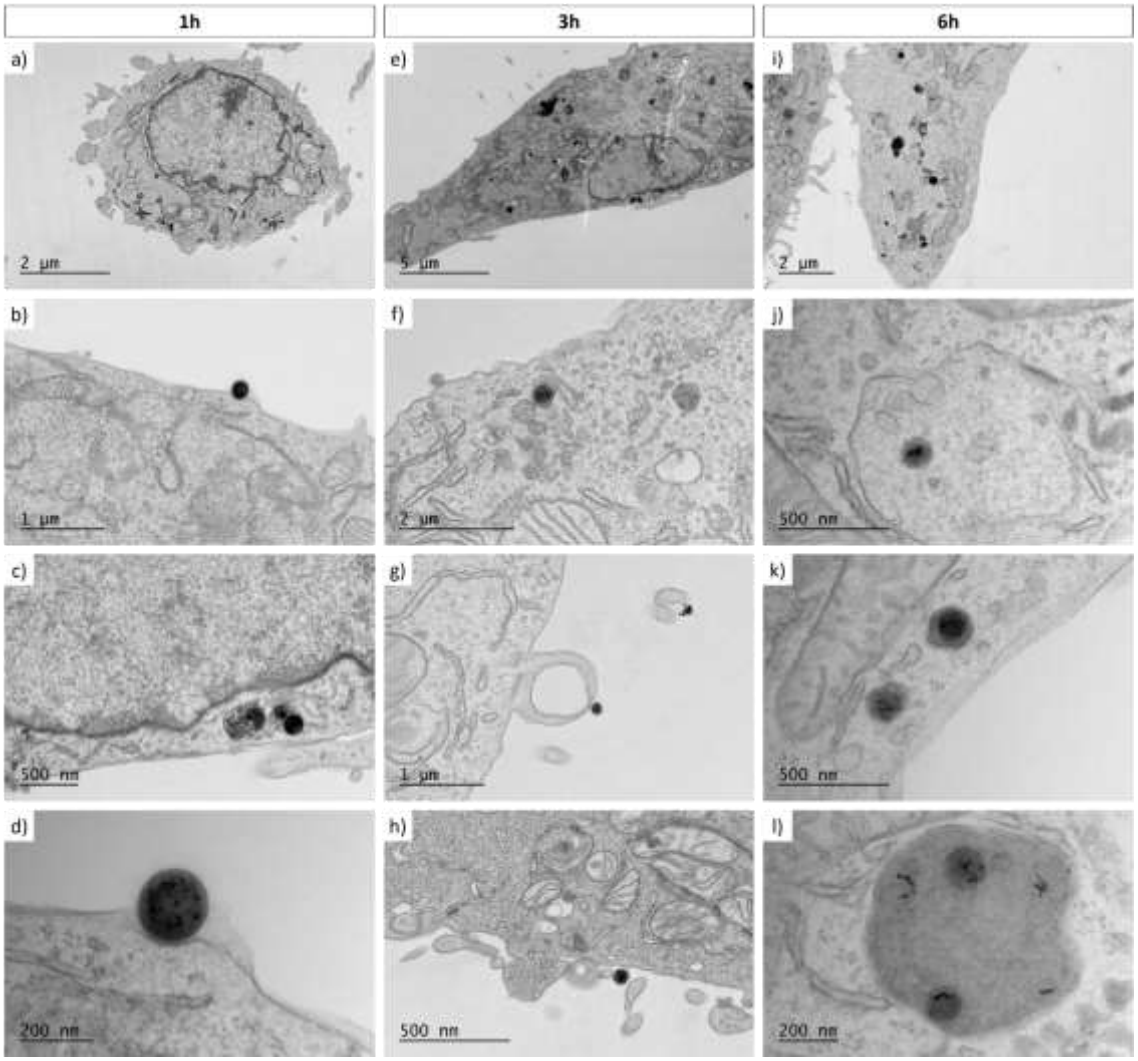


Figure 17 TEM pictures of RAW 264.7 cells incubated with pfNPs at the concentration 5 µg/ml a), b), c) and d) for 1 h , e), f), g) and h) for 3 h, and i), j), k) and l) for 6 h. The samples after incubation were fixed, embedded in epoxy resin and cut in thin slices over the TEM grids.

7.5 Immunostimulation Studies

After interaction with a nanovaccine, DCs/APC should mediate three critical functions; (i) uptake of extracellular tumor-antigens and further processing to produce MHC-antigen complexes, (ii) migration into lymph node presenting the antigen to the T cells, and finally (iii) expression of co-stimulatory receptors, such as CD80, CD86, in concert with pro-inflammatory cytokines such as IL-12(283). Those molecules are important in the priming of naive T cells by APC and in the further activation and

differentiation of effector T cell(284). Antigens administered in the absence of costimulatory molecules may not be enough to stimulate the immature DCs, which in turn will trigger the development of regulatory T cells and anergic T cell (intrinsically functionally inactivation)(285). The expression of CD80 and CD86 molecules in the RAW-264.7 cells was evaluated via staining, using fluorescent antibodies, followed by flow cytometry analysis (*Figure 18*).

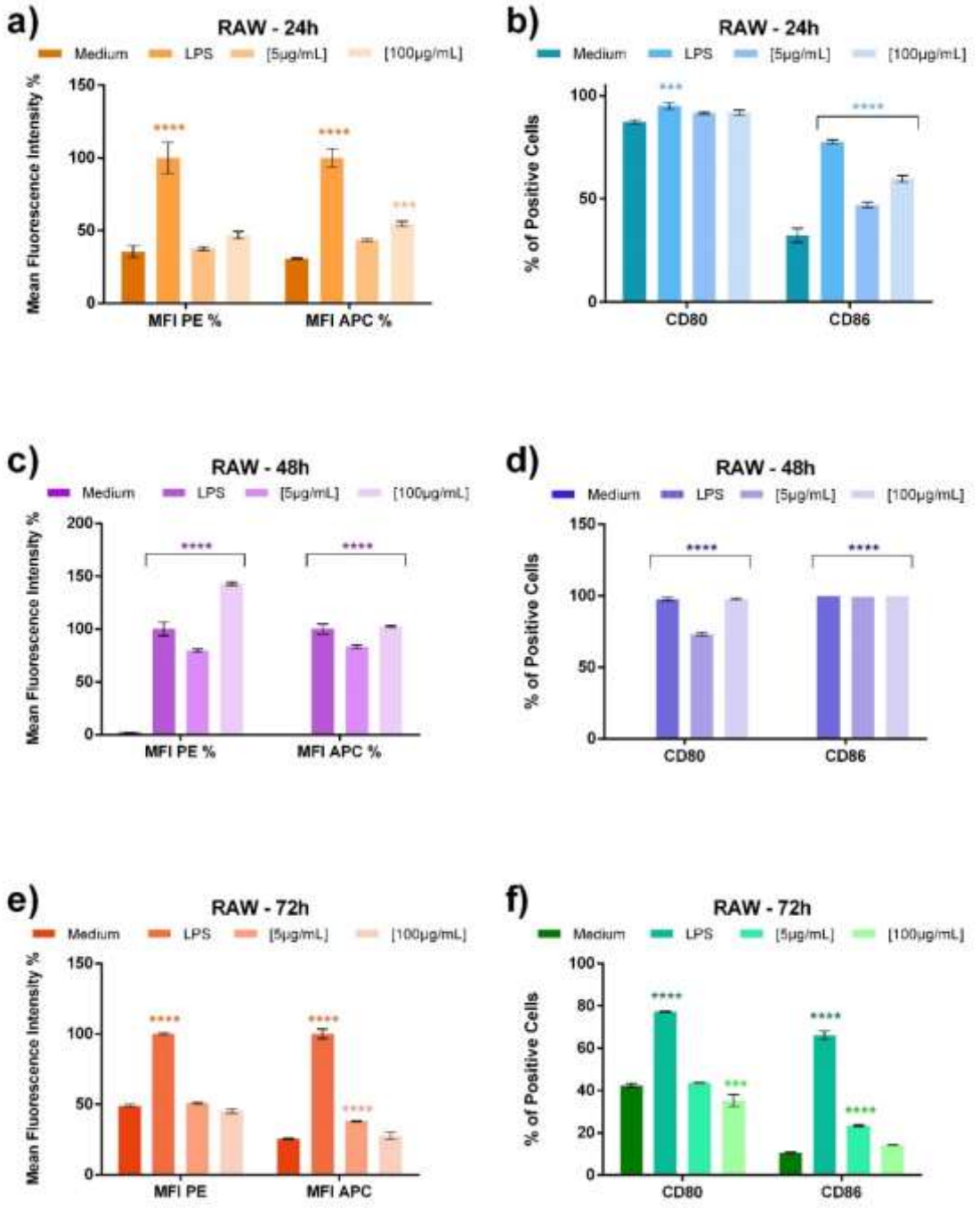


*Figure 18 Mean fluorescence intensity (%) and % of positive cells expressing CD80 and CD86 molecules after incubation with the pfNPs at different concentrations (5 and 100 µg/mL) to evaluate the immunostimulation at three time points a) and b) 24 h, c) and d) 48 h, and e) and f) 72 h. CD80 and CD86 were detected with Phycoerythrin-antiCD80 and Allophycyanin-antiCD86 antibodies, respectively. Complete medium and LPS were used as negative and positive controls, respectively. The samples were analysed with one-way ANOVA. The significance was assessed comparing to the negative control. The levels of significance were set by *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 probabilities. Error bars represent mean ± S.D. (n = 3).*

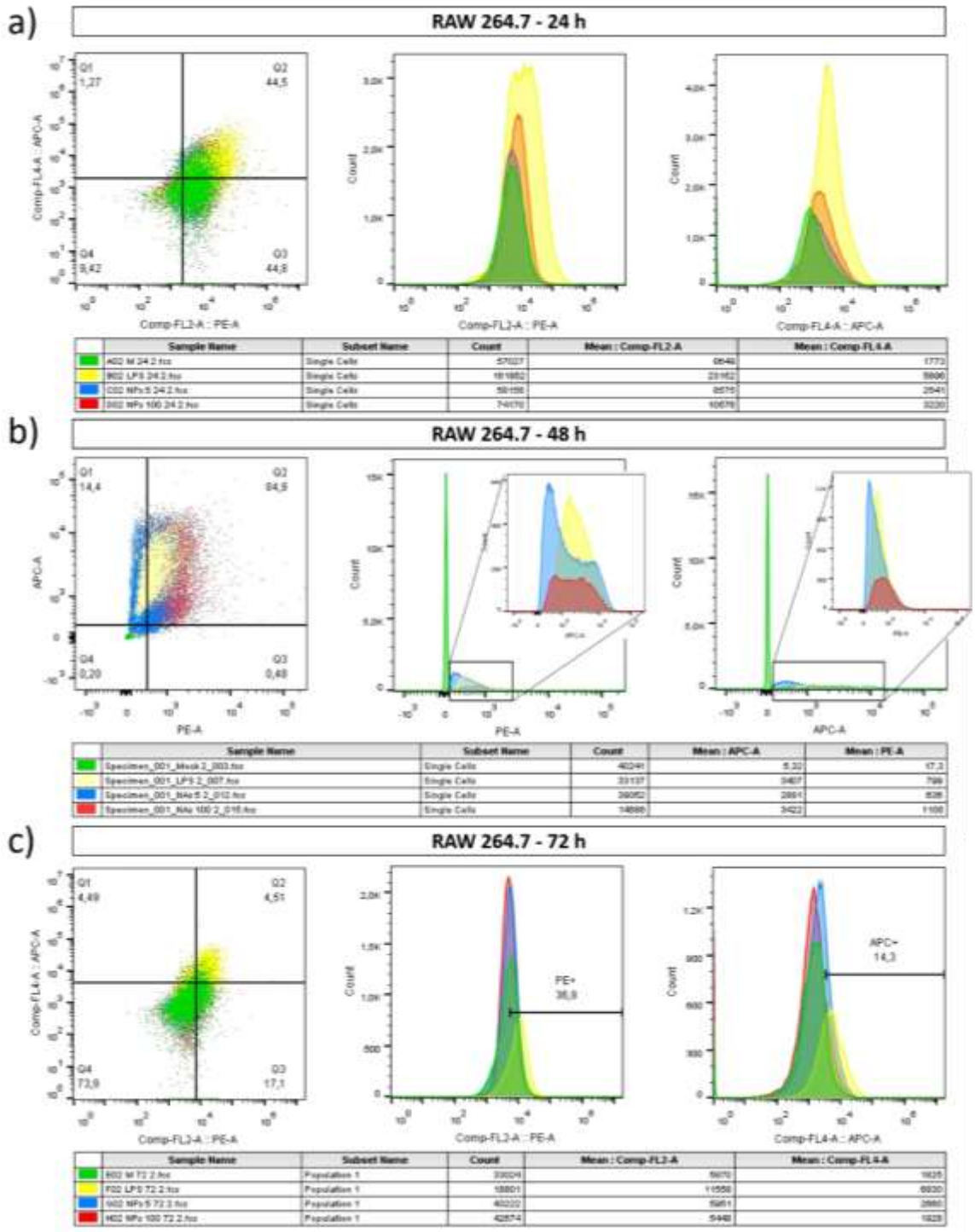


Figure 19 Flow cytometry histograms representing the expressions of CD80 and CD86 in RAW 264.7 cells after being incubated with pfNPs at different concentrations (5 and 100 µg/mL) to evaluate the response of immunostimulation in three time points a) 24 h, b) 48 h and c) 72 h. CD80 and CD86 were detected with Phycoerythrin-antiCD80 and Allophycyanin-antiCD86 antibodies, respectively. Complete medium and LPS were used as negative and positive controls, respectively.

The induction of the co-stimulatory signals by pfNPs at different concentrations (5 and 100 µg/mL) was evaluated at three time points; 24 h (*Figure 18-a–b*), 48 h (*Figure 18-c–d*) and 72 h (*Figure 18-e–f*).

After 24 h of incubation with pfNPs, the samples at concentration 100 µg/mL show statistically significant higher values of MFI APC (%) (***p < 0.001), and higher expression of CD86 compared with negative control at both 5 and 100 µg/mL concentrations (*Figure 18-a–b*). After 48h, an increasing value for MFI PE/APC (%) and % of CD80+/CD86+ cells compared with the respective controls (****p < 0.0001) suggested an enhanced nanoparticle-mediated immunostimulation (*Figure 18-c–d*). After 72 h of incubation with pfNPs induced statistically significant higher values of MFI APC (%) and higher expression of CD86 (****p < 0.0001) compared with negative control only on the samples incubated at the concentration of 100 µg/mL.

The higher expression of CD80 and CD86 at 48 h is also visible on flow cytometry histograms (*Figure 19-b*) and lower expression at 24 and 72h compared with its controls (*Figure 19-a–c)*. According to these results, the stimulation phenomena induced by pfNPs on RAW 264.7 cells is a time-independent and concentration-independent stimulation, displaying the best results at 48 h.

To date, no study evaluating the immunostimulation profile of silica-based or silicon-based NPs using RAW 264.7 cells is available to compare with the results of this study.

8. Conclusions

In this work, a careful review of the literature concludes that immunotherapy represents a possible key for the treatment of numerous types of cancers. In order to improve the efficiency of immunotherapy, nanotechnology can provide different solutions.

The experimental section consisted of the evaluation of pfNPs as potential nanocarriers for the development of cancer nanovaccine.

The stability assay showed that this NPs are quite stable, regarding their hydrodynamic diameter and ZP in various media, such as 10% of FBS DMEM, PBS (1×) and Human FFP24:PBS-EDTA 1:1 over 120 min. The cytocompatibility, assessed in three different cell lines, showed that pfNPs were nontoxic at 48 h up to 100 µg/mL for RAW 264.7 and KG-1 macrophages, as well as up to 10 µg/mL for BDCM cells. Furthermore, the uptake assay performed in two cell lines showed high uptake in RAW 264.7 and limited uptake of pfNPs in KG-1. In the last assay, the pfNPs' immunostimulatory properties were evaluated in RAW 264.7, concluding that the NPs induced a concentration/time-independent stimulation, displaying better results at 48 h.

The next steps in the work will include an assessment of various NPs' properties, such as the evaluation of the immune stimulation in human cell lines and in primary immune cells. Moreover, a thoroughly investigation should be conducted on the of the core particles (*e.g.*, gold, iron oxide, and PLL) on immunostimulation. Then, an antigen or antigenic source should be added to the pfNPs before evaluating their preventive and therapeutic vaccination efficacy *in vivo*. Finally, possibility of a combo-therapy with immune checkpoint inhibitors or traditional chemotherapeutics should be evaluated *in vivo*.

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