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Gold nanoparticle-based platforms for vaccine development

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Since their discovery, therapeutic or prophylactic vaccines represent a promising option to prevent or cure infections and other pathologies, such as cancer or autoimmune disorders. More recently, among a number of nanomaterials, gold nanoparticles (AuNPs) have emerged as novel tools for vaccine developments, thanks to their inherent ability to tune and upregulate immune response. Moreover, owing to their features, AuNPs can exert optimal actions both as delivery systems and as adjuvants. Notwithstanding the potential huge impact in vaccinology, some challenges remain before AuNPs in vaccine formulations can be translated into the clinic. The current review provides an updated overview of the most recent and effective application of gold nanoparticles as efficient means to develop a new generation of vaccine.

Introduction

Vaccination is one of the most cost-effective strategies to prevent death and morbidity associated with infectious diseases, given the induction of pathogen-specific humoral immune responses. Over the past years, vaccines have been one of the many crucial medical advancements that has contributed to an increase in life expectancy, conferred long-term protective immunity to the population and saved millions of lives and the eventual eradication of some viral and bacterial infectious diseases [1,2].

Vaccines prime the immune system to generate an adaptive response, characterized by cellular ($CD4^+$ or $CD8^+$ T-cells) or antibody responses. Therefore, immunomodulation is in the forefront of the development of treatments for many pathologies. The immune system can be stimulated or suppressed to help in the fight against infectious diseases or other kind of pathologies (i.e. cancer or autoimmune diseases) [3,4]. Vaccines can consist of live-attenuated or inactivated pathogens, purified proteins, or subunits based on small antigens (i.e. peptides or carbohydrates) that should elicit specific immune responses. With respect to the use of whole pathogen vaccines, subunit vaccines are usually safer to administer, but they are generally poorly immunogenic. To overcome such issues, alternative approaches can be taken, such as (1) the use of adjuvants (e.g. alum, squalene, toll-like receptors (TLRs) agonists, monophosphoryl lipid A) that can be added to vaccine formulations in order to stimulate the immune system [1]; (2) conjugation of the antigen to an immunogenic carrier (i.e. immunogenic proteins tetanus toxoid (TT), diphtheria toxoid (DT) and its genetically detoxified form (CRM_{197})) [2] or (3) use carriers which can display multiple copies of antigens, evoking the multivalent effect and enhancing the immune response [3,5].

Nanotechnology represents a cutting-edge approach, able to address some of the most relevant vaccinology concerns. Nanomaterials (NMs) with sizes between 1 and 100 nm (i.e.,

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in the range of cellular components and microorganisms) have emerged as innovative tools in many biomedical applications, from diagnostics to drug delivery. Many NMs are characterized by high biocompatibility, morphological customization, reliable functionalization, and prolonged circulation in blood [6,7]. NMs can be internalized by antigen presenting cells (APCs, such as dendritic cells, DCs, or macrophages) via endocytosis, stimulating the immune systems by releasing cytokines that initiate the adaptive immune response (Fig. 1) [6,8,9]. Subsequently, DCs can migrate to the lymph nodes where they interact with T-cells and present non-self-peptides on major histocompatibility complexes (MHC) class I or II, producing cytokines and driving antigen-specific $CD8^+$ or $CD4^+$ T-cell activation [9].

In the interaction between immune system and nanomaterial, several crucial aspects need to be taken into consideration. To generate a biased immune response, particle size, surface charge and shape must be modulated in a rational manner to facilitate the crossing of the physiological barriers and the access to immune cells. Some excellent and recent reviews report critical surveys about the modulation of the immune response by means of different kind of nanomaterials [3,4,9,10].

Among many different nanoparticles which include lipids, self-assembled proteins, biopolymers and inorganic materials [5], properly functionalized gold nanoparticles (AuNPs), represent one of the most promising nanomaterial in the development of a new generation of vaccines [7,11]. In the present paper we will review the most recent results (see Table 1 for a complete summary) that evidence the potential of AuNPs as innovative nano-vaccines platforms.

Gold nanoparticles in vaccinology

AuNPs have aroused huge interest in vaccinology, due to their reliable surface functionalization, biocompatibility, size and shape customization, and optical properties. Moreover, owing to their inertness, AuNPs can be exploited both as delivery agents and as adjuvants, with the ability to extensively enhance immune responses while assuring minimal toxicity [1].

AuNPs are usually synthesized by a reductive reaction of chloroauric acid ($HAuCl_4$), using well-established Turkevich or Brust-Schriffins procedures followed by surface modification and stabilization (Fig. 2). Depending on the desired size and morphology, the precursor Au^{III} salt is reduced to Au^0 by means of sodium borohydride, sodium citrate, or ascorbic acid with the eventual presence of templating agent (i.e. cetyl trimethylammonium bromide) [11,12]. Despite their inertness, AuNP surface can be functionalized by forming stable bonds with sulfur-containing compounds or by multilayer coating. This relatively straightforward functionalization confers AuNPs great versatility, extending their use into the biomedical field [11–13]. When designing a nano-gold platform, size and shape have a crucial impact in terms of

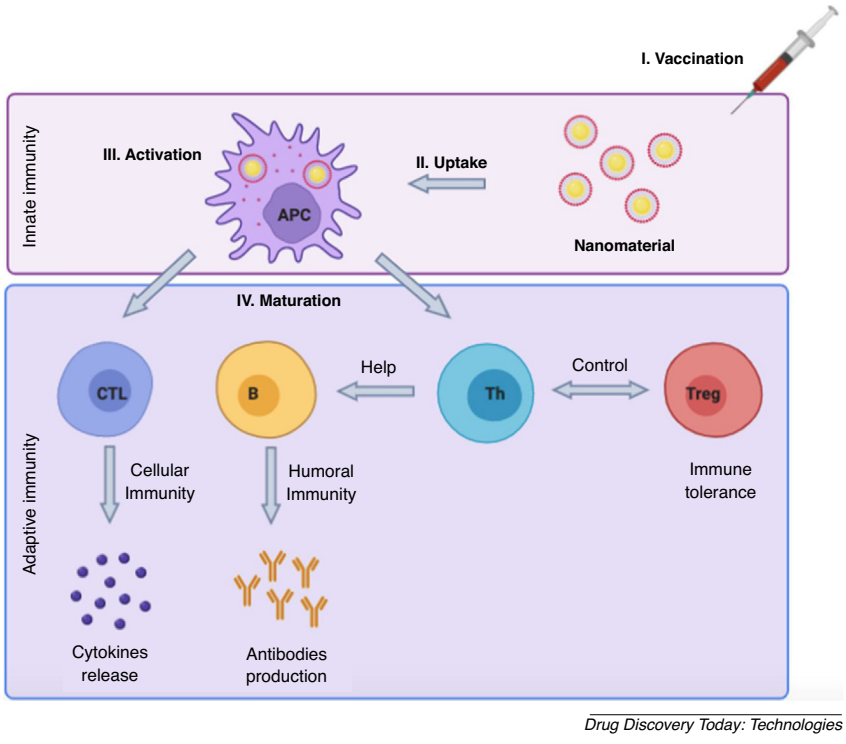

Fig. 1. Schematic representation of innate and adaptive immune responses upon exposure to nanomaterial-based vaccines. APCs process the antigens and promote T cell proliferation towards cellular (cytokines), humoral (antibodies) or tolerogenic responses. APC, antigen presenting cell; CTL, cytotoxic T cells; B, B cells; Th, T helper cells; Treg, regulatory T cells.

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Table 1. Complete summary of the most recent applications of AuNPs in vaccinology, their morphology, and their field of applications.

AuNP (morphology)	Application	Surface functionalization	Experiment	Ref
15 nm (sphere)	Adjuvant	$Flagellin_{(1-161)}$	*In vivo* (injected)	[35]
20 nm (sphere)	Adjuvant	SIINFEKL and polyIC	*In vivo* (injected)	[13]
20 and 41 nm (sphere)28 × 7.9 nm and 41 × 12 nm (rods)	Adjuvant	PolyIC(40:400)	*In vivo* (intranasal)	[15]
70 nm (nanostar)	Adjuvant	–	*In vivo* (injected)	[45]
2 nm (sphere)	Bacterial infections (*S. pneumoniae*)	Oligosaccharide epitopes *S.pn*14/$OVA_{323-339}$	*In vivo* (injected)	[25]
2 nm (sphere)	Bacterial infections (*S. pneumoniae*)	Oligosaccharide epitopes *S.pn*14 and 19F	*In vivo* (injected)	[26]
15.5 nm (sphere) 51.2 nm (nanourchins)	Bacterial infections (*GAS*)	Tetra and hexarhamnosides	ELISA	[27]
15 nm (sphere)	Bacterial infections (*B. pseudomallei*)	Hemagglutinin/FlgL/LPS	*In vivo* (intranasal)	[28]
15 nm (sphere)	Bacterial infections (*EHEC*)	LomW/EscC	*In vivo* (injected)	[31]
2 nm (sphere)	Bacterial infections (Listeria)	LLO 91–99/GADPH 1–22/Advax^{TM}	*In vivo* (injected)	[32–34]
2 nm (sphere)	Viral infections (HIV)	(oligo)mannosides/carboxyl-ending linker	*In vivo* (injected)	[40]
2.3 nm (sphere)	Viral infections (HIV)	Gagp17/dimanoside (Manα1–2Man)	*Ex vivo*	[41]
12 nm (sphere)	Viral infections (Influenza)	M2e protein	*In vivo* (intranasal)	[43]
18 nm (Sphere)	Viral infections (Influenza)	H3N2/HA/FliC	*In vivo* (intranasal)	[44]
3 nm (sphere)	Cancer	OVA protein	*In vivo* (injected)	[47]
3–5 nm (sphere)	Cancer	TF disaccharide	*In vivo* (injected)	[49]
5–20 nm (sphere)	Cancer	Tn (α-GalNAc)	*In vivo* (injected)	[51]
7 and 14 nm (sphere)	Cancer	MUC-1 glycopeptides	*In vitro (quartz crystal microbalance and dot-blot Immunoassay)*	[52]
15 nm (sphere)	Cancer	MUC-1 glycopeptides	*In vitro*	[53]
22–30 nm (sphere)	Cancer	MUC-1 glycopeptides/CD4 T-cell epitope	*In vivo*	[54]
30 nm (sphere)	Cancer	SIINFEKL OVA peptide	*In vivo* (injected)	[55]
24 nm (Sphere)	Cancer	pCMV-MART1	*In vivo* (injected)	[56]
17 nm (sphere)	Cancer	PD-L1 siRNA/STAT3 siRNA	*In vivo* (injected)	[57]
45 x 15 nm (rods)	Cancer	IDO siRNA	*In vivo* (injected)	[58]
30 nm (sphere)50 nm (star)60 nm (cage)30–40 nm (prism)	Parasite Infections (*P. falciparum*)	CHrPfs25	*In vivo* (injected)	[63]
60 nm (sphere)	Non-infectious diseases (Diabetes)	AhR/proinsulin/ITE	*In vivo* (injected)	[65]
2–4 nm (sphere)	Non-infectious diseases (Diabetes)	Proinsulin (PI_{C19-A3})	*In vivo* *Ex vivo*	[66]

antigen presentation, cellular uptake, blood clearance, biodistribution and immunological response [14]. AuNPs, in fact, up-regulate the expressions of pro-inflammatory cytokines via different pathways depending on their geometry and mode of administration [3,6,9,10,15]. Their size mostly affects the uptake and biodistribution whereas their surface functionalization can alter the circulation time [16]. All these variables are strongly interrelated and even though several authors have addressed this paramount topic, no systematic approach has yet been found regarding the best preferred size and shape for every application [6,17,18]. Moreover, the functionalization step plays a pivotal role considering that, when exposed to biological media, AuNPs can be coated by proteins and other biomolecules forming the so-called 'biocorona' [19,20]. The formation of such a bio-corona can have major effects on the biodistribution and NP targeting activity, since the original functionalization can be hidden. Another crucial aspect that must be considered is the AuNP electronic properties [7,12]. These noble metal NPs can absorb and convert electromagnetic radiation into heat and, thus, they

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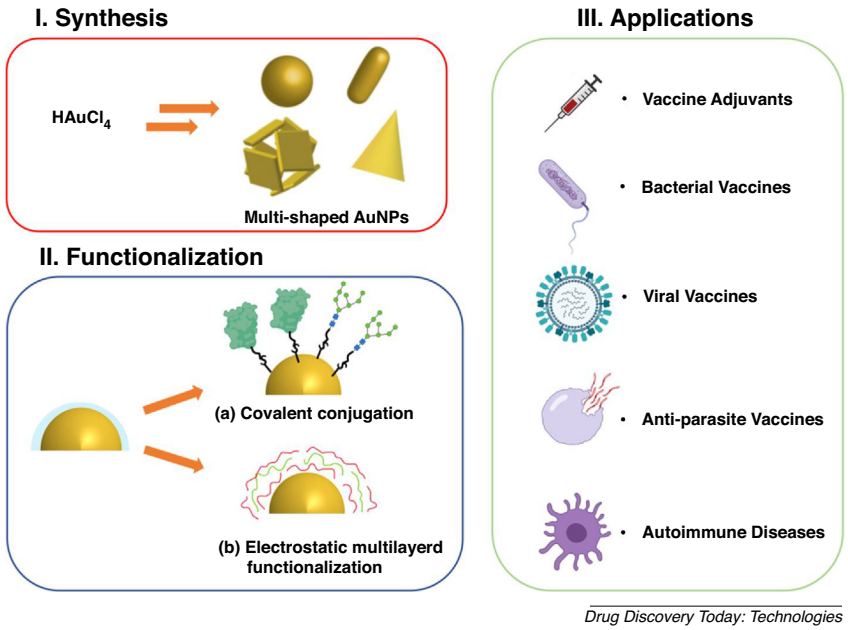

Fig. 2. I. Synthesis: AuNPs can be synthesized from Au^{III} salts, finely tuning size, and shape. **II. Functionalization: a.** Reliable Au-S chemistry is used to functionalized gold surface with ligands, proteins and oligo or polysaccharides. **b.** Surface functionalization can be accomplished by a multilayered strategy. **III. Applications:** a vast number of different fields can have great advantage using AuNPs: they will be reviewed in their most recent progress in this paper.

can be exploited for targeting and therapeutic purposes. Notably, the application of AuNPs in nanomedicine covers several fields from drug delivery to diagnostics and therapeutics, making these nanomaterials extremely versatile and promising.

In the present paper, the most recent examples of AuNP exploitation against bacterial infections, viral infections, cancer, parasite infections and non-infectious diseases, will be reviewed.

Bacterial infections

Diseases caused by bacterial infections can vary from mild symptoms to lethal sepsis. Such infections can have a strong impact on public health, worsened by a long list of antibiotic-resistant bacteria for which new and efficient preventive therapies are urgently needed [2,21]. Bacteria cell envelopes are characterized by a dense array of glycans: Gram-negative bacteria are coated by a peptidoglycan wall surrounded by a lipopolysaccharide (LPS) membrane, while Gram-positive lack the outer lipid membrane and present only a thick polysaccharide layer. These polysaccharides protect the bacteria and are the main way of interaction of the pathogens with the host immune system, representing the major virulence factor and thus, the optimal target for vaccine design. Nevertheless, it is well known that the major drawback of polysaccharide-based vaccines is the limited immunogenicity (especially for infants, elderly and immunocompromised patients) due to the induction of T cell-independent immune responses. This issue can be overcome by conjugating a fragment of the capsular polysaccharide to an immunogenic carrier protein (i.e. TT, DT, CRM_{197}) able to stimulate B cell maturation to memory cells. Such successful strategy paved the road for the development of promising new carbohydrate-based vaccines, comprised of natural polysaccharides or well-defined synthetic oligosaccharides [2,22]. Nevertheless, some drawbacks related to immune interference phenomena, referred to as carrier epitope suppression, can lead to a reduction of the anti-carbohydrate immune response against glycoconjugate formulations. Therefore, a continuous effort is devoted to the development of new protein carriers or their replacement with shorter peptides or innovative nano-carriers [2,22,23]. In this contest to enhance the poor immunogenicity of carbohydrate antigens, AuNPs offer many advantages and represent optimal and versatile platforms [5,24]. The controlled surface functionalization can afford a well-mimic of the natural glyco-cluster, leveraging the multivalent effect to induce an enhanced immune response. AuNPs have been used as vaccine candidates against *Streptococcus pneumonia* by coating 2 nm AuNPs with different ratios of the branched tetrasaccharide unit β-D-Galp-(1–4)-β-D-Glcp-(1–6)-[β-D-Galp-(1–4)-]β-D-GlcpNAc-(1–) of *S. pneumonia* capsular polysaccharide serotype 14 and a T-helper peptide, ovalbumin 323–339 peptide ($OVA_{323–339}$) [25]. The simultaneous coating of the saccharide antigen and a T-helper peptide was essential to trigger anti-saccharide antibodies and avoid the epitope suppression issue. Moreover, the same nanoparticle surface can display different type of antigens and immunogenic peptides or adjuvants

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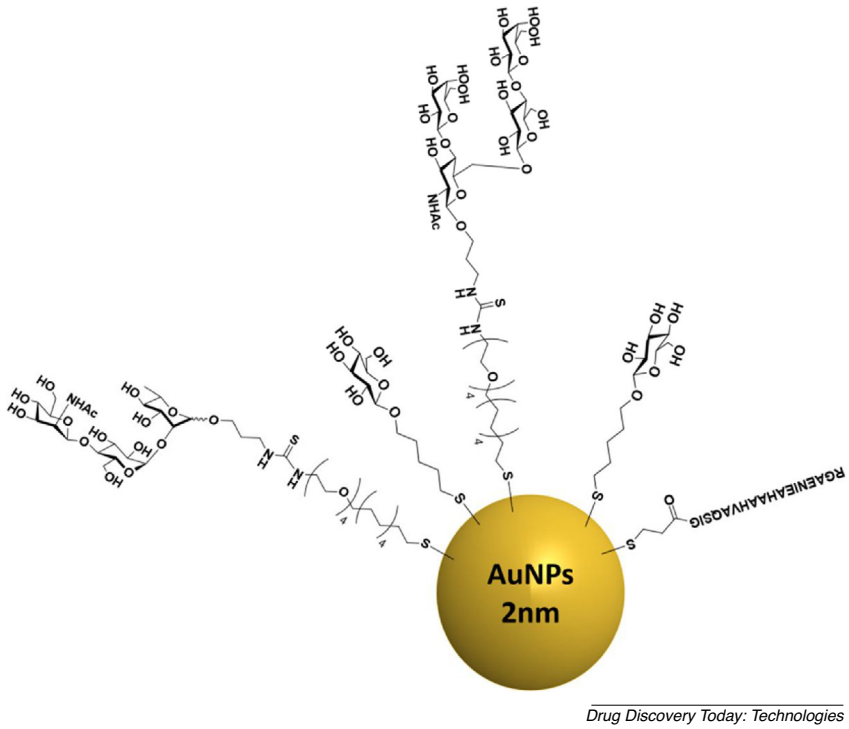

Fig. 3. AuNPs functionalized with 4 component comprised of (1) synthetic tetrasaccharide epitope of *S. pneumoniae* serotype 14 (β-D-Galp-(1–4)-β-D-Glcp-(1–6)-[β-D-Galp-(1–4)-]β-D-GlcpNAc-(1–)); (2) synthetic trisaccharide epitope of *S. pneumoniae* serotype 19F (β-D-ManpNAc-(1→4)-α-D-Glcp-(1→2)-α-L-Rhap-(1→]); (3) a T-helper ovalbumin 323–339 peptide to evoke anti-saccharide antibodies; (4) D-glucose as inner component to ensure water solubility.

simultaneously. In a recent example, 2 nm AuNPs were functionalized with four components to develop a candidate against different serotypes of *S. pneumoniae*, a major pathogen causing pneumonia with over 2 million deaths annually (Fig. 3) [21,26]. AuNPs were engineered with: (1) two synthetic oligosaccharide epitopes of *S. pneumoniae* serotype 14 (the tetrasaccharide unit) and 19F (trisaccharide (β-D-ManpNAc-(1→4)-α-D-Glcp-(1→2)-α-L-Rhap-(1→]); (2) a T-helper ovalbumin 323–339 peptide; (3) D-glucose. After immunization in mice, glyconanoparticles induced immune response primarily directed towards *S. pneumoniae* 14 comparable with the commercially available PCV13 vaccine [26]. This result emphasizes the exciting advantages arising from an accurate design and synergistic action of AuNP surface functionalization. Recently, our group preliminary evaluated the role of AuNP geometry designing oligorhamnan-functionalized NPs to address infections by a Gram-positive Group A Streptococcus (GAS), a bacterium responsible for a large range of infections in developing countries. Spherical and urchinated AuNPs functionalized with the synthetic tetra and hexarhamnosides were able to inhibit the binding of specific polyclonal serum much better than the unconjugated oligosaccharides, regardless the different morphologies [27].

Burkholderia pseudomallei is a Gram-negative bacterium, common to tropical and subtropical climates, that causes melioidosis in humans and other mammals. The infection from *B. pseudomallei* presents a variety of clinical manifestations, ranging from soft tissue abscesses to fulminant sepsis. Currently, melioidosis treatments are limited because the bacterium is inherently resistant to antibiotics and no vaccines are available or in advanced clinical trials. Recently, a reverse vaccinology approach has been successfully applied to identify new immunogenic *B. pseudomallei* proteins: hemagglutinin and FIgL. Such proteins have been incorporated into a nanoglycoconjugate vaccine formulation based on 15 nm AuNP already coated with highly antigenic capsular lipopolysaccharides (LPS) of the bacteria [28]. The same research group previously demonstrated that the incorporation of LPS onto AuNPs showed significant protection in murine and nonhuman primate models of inhalational glanders, enhancing the immune response and increasing the protection against the closely related pathogen *B. mallei* [29,30]. The latest and promising results based on 15 nm glyco-AuNPs with the new immunogenic proteins evidenced that immunized animals generated both antigen- and LPS-specific IgG titers in mice. Additionally, immunization demonstrated between 90% and 100% survival following a lethal challenge with *B. pseudomallei* [28].

A reverse vaccinology strategy has been exploited also to identify two new immunogenic proteins, LomW and EscC, that have been covalently linked on AuNP surface to challenge enterohemorrhagic *Escherichia coli* (EHEC) O157:H7, a

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human pathogen that causes diarrhea and hemorrhagic colitis worldwide [31]. Since antibiotic treatment is contra-indicated, vaccination represents the best alternative to prevent such infections. Proteins were conjugated through a thio-linker to 15 nm AuNPs and injected subcutaneously into mice; the new systems elicited strong humoral responses with high titer of IgG and sIgA towards each antigen.

AuNPs represent a promising alternative to develop a new type of vaccine against listeriosis, induced by *Listeria monocytogenes* (Listeria), a bacterium that causes opportunistic infections in immuno-compromised patients and cerebral listeriosis in fetuses and newborns. AuNPs were functionalized with two peptides from Listeria's virulence factors, listeriolysin O (LLO) 91–99 and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) 1–22 peptide antigens. In both cases peptide-functionalized AuNPs were extensively studied and proved their efficacy *in vitro* and *in vivo*, inducing $CD8^+$ T-cell immunity, response enhanced by the co-formulation with Advax^{TM}, a carbohydrate-based adjuvant derived from microparticles of delta β-D-[2–1]poly(fructo-furanosyl)α-D-glucose (delta inulin) [32,33]. Moreover, GADPH-AuNPs demonstrated their ability to pass the placenta barrier and to protect neonates infected through vertical transmission of the bacteria, inducing high level of IL-12 and a decrement of IL-6 in newborns from vaccinated mothers [34].

Recently, AuNPs were favorably used also against *Pseudomonas aeruginosa*. AuNPs conjugated to N-terminal domains of *P. aeruginosa* flagellin (flagellin(1–161)) elicited high titers of anti-flagellin IgG antibodies able to recognize and neutralize the gram-negative opportunistic bacterium in mice models [35].

Viral infections

The actions against viral infections have been at the center of scientific research for centuries and the development of efficient vaccines able to induce specific immunity and long-lasting memory remains the main goal in this fight. Almost four decades after its discovery in 1983, human immunodeficiency virus (HIV) continues to be a major global public health concern. The huge potential to induce mucosal immunity makes AuNPs ideal platforms for intranasal applications and for topical treatment, designing vaccines against sexually transmitted diseases such as HIV. Nowadays there is only one HIV vaccine in human clinical trials (RV144), and the progress has been hampered given the lack of realistic animal models to validate the nanovaccines [36]. Antiretroviral (ART) therapies have proven to fight infection, prolong the life-expectancy of infected individuals and lower the risk of infection. However, they do not provide a cure and individuals taking this lifelong medication must overcome issues associated with side effects, drug resistance, strict dosing regimens and high costs, which makes this strategy unfeasible for developing countries. Therefore, an effective HIV vaccine is urgently needed to prevent infection and progression of the acquired immune deficiency syndrome (AIDS). Among the many challenges to achieve this goal, one of the greatest is HIV diversity, reflected by the presence of subtypes, circulating recombinant forms, and continuous viral evolution to evade the host immune system [37]. Hence, effective vaccines need to (1) elicit broadly neutralizing antibodies (bNAbs) capable of neutralizing the majority of circulating HIV strains and (2) generate strong $CD4^+$ and $CD8^+$ T-cell responses able to kill virally infected cells without representing a hazard. Most vaccine developers have focused on envelope glycoproteins (Env) on the viral membrane, which are the sole target for bNAbs against HIV. However, despite decades of efforts, there is no a vaccine able to induce sufficient bNAbs and generate protective immunity [37,38]. AuNPs aim to overcome current limitations by inducing a synergistic humoral and cellular immune response. The similar structures of the 'antennas' constituted by high-mannose N-glycans present on the HIV-1 envelope glycoprotein gp120 were proposed by simultaneously incorporating tetra- and penta-mannosides on AuNP surface. These glycans not only constitute an epitope for the HIV broadly neutralizing antibody 2G12, but also target DC-SIGN receptors involved in the first steps of HIV infection. Mannose-AuNPs, *in vitro*, inhibited HIV gp120 binding to DC-SIGN and eliciting IgG 2G12 [39]. (Oligo)mannoside AuNPs have been also leveraged to control the conformation of the V3 loop peptide, derived from the third variable region of HIV gp120 protein, the major immunogenic domain of HIV-1 [40]. Displaying on the same nanoparticles both (oligo)mannosides and a carboxyl-ending linker, the negative charged nanoplatforms were able to stabilize either the α-helix or β-strand V3 conformation. Rabbits immunized with such AuNPs successfully triggered antibodies able to recognize a recombinant gp120. To enhance the poor immunogenicity of the high mannose oligosaccharides, AuNPs carrying simultaneously di-mannose and Gag p17, a HIV-peptide antigen, were synthesized. *Ex vivo* experiments with monocyte-derived dendritic cells (MDDCs) and lymphocytes from HIV-1 infected patients showed that such AuNPs increased HIV-specific $CD4^+$ and $CD8^+$ proliferation and induced highly functional cytokine secretion compared with free peptides. Altogether, results suggest that these formulations not only induce a T_H1 response but also promote a combined T_H1/T_H2 profile which could activate B cells enhancing the humoral arm of the immune system. Thus, co-delivery of HIV-1 antigens by AuNPs could induce not only HIV-specific cellular response but also an antibody response, like that exerted by the broadly neutralizing 2G12 antibody [41].

Great efforts are dedicated to the identification of a universal vaccine against seasonal influenza, which puts great economic pressure on public healthcare systems worldwide. Unfortunately, a new influenza vaccine needs to be

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developed every year according to the new circulating strains. Therefore, a universal influenza vaccine focused on type-specific amino acid sequences and conformational epitopes would be highly desirable [42]. In a preliminary study, 12 nm AuNPs were functionalized with the highly conserved extracellular region of the matrix 2 protein (M2e) of influenza and intranasally injected in mice, inducing M2e-specific IgG serum antibodies. The co-administration of AuNPs and CpG, as adjuvant, strongly stimulated M2e-specific IgG antibodies, inducing cross-protection against diverse influenza viral strains [43]. A more recent approach is instead based on 18 nm AuNPs conjugated with recombinant trimetric A/Aichi/2/68 (H3N2), hemagglutinin (HA) and TLR5 agonist flagellin (FliC) [44]. Combining the intranasal *in vivo* administration and *in vitro* results, the conjugated AuNPs resulted able to effectively induce a strong immune response by activating DCs and T cells.

Polyelectrolyte self-assembled AuNPs proved to be very efficient as innovative nanoplatforms. Zhang et a. prepared multilayered, self-assembled AuNPs by alternate deposition of polyinosinic-polycytidylic acid (polyIC) and $OVA_{257–264}$ octapeptide SIINFEKL [13]. The former is an anionic nucleic acid TLR3 agonist with strong adjuvant properties whereas the latter acts as a peptide antigen acting as cationic anchor. These nanoparticles were efficiently internalized by primary dendritic cells, resulting in activation and presentation of the antigens, promoting high levels of antigen-specific $CD8^+$ T cells response, compared to mice treated by co-administration of antigen (SIINFEKL) and adjuvant (polyIC). In another example, a low-molecular-weight synthetic RNA adjuvant, polyinosinic-polycytidylic acid (uPIC(40:400)), was used to electrostatically coat spherical and rod-shaped AuNPs [15]. The needle-free intranasal administration of uPIC(40:400)-gold nanorods together with influenza virus hemagglutinin showed an enhanced adjuvanticity of uPIC(40:400), leading to the suppression of *influenza* viral infection in mice while maintaining low inflammatory cytokine production. Interestingly, a shape-dependent adjuvant effect of AuNPs has been reported in another contribution in which the addition of gold nanostars to virus-like particle nanoformulations powerfully activated macrophages and induced specific immune response against foot-and-mouth disease (FMD) [45].

Cancer

In 1893, W. Coley postulated for the first time that the immune system was able to recognize and set a response against tumors, paving the way towards the development of cancer vaccines as a potential alternative to conventional cancer therapies [46]. As opposed to prophylactic vaccines to prevent infectious diseases, therapeutic cancer vaccines work to activate or modulate the body's immune response against malignancies. Cancer cells express a series of tumor-associated antigens (TAAs), to which cytotoxic T cell responses are difficult to induce. Thus, the effectiveness of current vaccination approaches is limited by the weak immune-stimulating capacity of tumor antigens. AuNPs constitutes a smart template for the multivalent presentation of TAA antigens, co-delivery of antigens and adjuvants and has proven to act as an efficient adjuvant, enhancing immunogenicity [47]. Tumor-associated carbohydrate antigens (TACA's) are glycan chains specifically and aberrantly expressed on the surface of malignant cells and thus, are considered promising targets for cancer immunotherapy [48].

Mucins are a family of aberrant proteins highly overexpressed on many tumor cells. They primarily exist as two domains: one large extracellular subunit and a smaller, transmembrane cytosolic domain. The extracellular domain consists of tandem repeat (TR) motifs of about 20 amino acids that contain a large degree of serine and threonine O-glycosylated. The most prevalent tumor associated carbohydrate antigens present on mucins are the Tn (GalNAcα-O-Ser/Thr), the Thomsen Friedenreich (TF) (Galβ1–3GalNAcα-O-Ser/Thr), and the sialyl-Tn (Neu5Acα2–6-GalNAcα-O-Ser/Thr) TACAs. A strong antibody response to these antigens leads to a better prognosis and less aggressive tumors. Among mucins, MUC-4, is sparse or absent on normal pancreas tissue but aberrantly expressed in pancreatic cancer making it an important target for solid tumors. Spherical 3–5 nm AuNPs were coated with tumor-associated glycopeptides consisting in the cell surface mucin MUC-4, functionalized with the TF disaccharide antigen at different sites. The subcutaneous immunization of mice, comprised of functionalized AuNPs and a peptide adjuvant derived from protein C3d, showed a small but encouraging and statistically significant immune response with production of both IgM and IgG isotypes [49]. Very recently, the results obtained using this nanoconstruct were exploited to produce a monoclonal antibody specific for the N-terminal glycosylated peptide domain that represents a novel immunogen target, interesting to develop diagnostic antibodies or immunotherapies [50]. A simple approach to the synthesis of anticancer vaccines without the need of a typical protein component is based on a polymeric version of the tumor-associated Tn monosaccharide antigen. By exploiting the reversible addition-fragmentation chain transfer (RAFT) polymerization, the polymers were conjugated to AuNPs, producing peptide-free nanoscale glycoconjugate. AuNPs, *in vivo*, induced a significant production of IgG antibodies, selective to the Tn antigen glycan and cross-protective immunity toward mucin glycoproteins displaying Tn antigens [51].

The tethered mucin glycoprotein MUC-1 is over-expressed and with a distinctly altered glycan pattern on epithelial tumor cells, therefore it represents a target structure in the development of effective carbohydrate-based cancer vaccines. Nevertheless, the natural glycopeptide antigen has low immunogenicity and a T-cell independent response, drawbacks that

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could be overcome by using multivalent carriers. Novel robust nanoplatforms able to successfully present MUC1-glycopeptide antigens multivalently to their corresponding antibodies have been studied [52]. Three different glycosylated MUC-1 partial structures with a full 20 amino acids, containing tandem repeats domain and a spacer (to guarantee flexibility and distance to the nanoparticle core) were synthesized using solid-phase peptide synthesis. Such glycopeptides structures were then used to coat the gold surface of 7 and 14 nm AuNPs, developing innovative and reliable MUC-1 functionalized gold colloids able to detect selectively primary anti-MUC1 antibody as proved by quartz crystal microbalance and by a dot-blot immunoassay. In another approach, MUC-1 was exploited to coat 15 nm AuNPs and used to treat monolayers of peritoneum-derived macrophages [53]. The MUC-1-AuNPs powerfully activated macrophages, releasing cytokines as TNF-, IL-6, IL-10, and IL-12. Moreover, a predominant M1 polarization of macrophages resulted after MUC-1-AuNPs exposure, representing an important achievement as M1 macrophages play a crucial role in antigen presentation and tumor vaccine generation. In another approach, PEGylated AuNPs (in a range from 22 to 30 nm) were functionalized with a glycopeptide sequence derived from MUC-1 covalently linked to CD4 T-cell peptide epitope (P30 from Tetanus Toxoid) [54]. Such nanosystems were evaluated and their ability to induce selective antibodies was preliminary tested *in vivo* on a small number of animals: after immunization, mice showed significant Th1 and Th2 mediated immune responses directed to the glycopeptide antigen.

Peptide antigens can also be used to induce an immunologic response: the coupling to AuNPs has a double advantage, preventing peptide degradation and allowing a targeted delivery to APCs. AuNPs were assessed for the delivery of an exogenous ovalbumin (OVA) peptide antigen to evaluate the therapeutic effect in a B16-OVA mice tumor model. The subcutaneous administration AuNP-OVA induced strong antigen-specific responses in mice and cytokine release in bone marrow-derived DC cultures. These findings support the use of AuNPs as effective peptide vaccine carriers with the potential of using lower and safer adjuvant doses during vaccination [55].

In recent years, AuNPs have demonstrated promising potential as vehicles for targeted delivery of nucleic acids given their ability to protect genetic material from degradation by nucleases. A recent study presented AuNPs covalently functionalized with a shikimoyl group as DC targeting ligand and a transfection enhancing guanidinyl ligand. AuNPs were electrostatically coupled with a melanoma antigen (pCMV-MART1) encoded DNA. The nanovaccine demonstrated the ability to deliver DNA to DCs *in vitro* and *in vivo* with high efficiency, inducing long-lasting protective immunity as well as therapeutic effects against murine melanoma [56]. Unlike DNA vaccines, RNA vaccines are less likely to cause side effects due to their rapid degradation and clearance. Short interfering RNA (siRNA) has shown promising potential as a molecular approach to down-regulate specific gene expression in cancer cells. A mechanism of tumor immune evasion consists in immune checkpoint interactions between ligands expressed on tumor cells (e.g. PD-L1) and receptors on the surface of T-cells (e.g. PD-1) to inhibit cytotoxic T-cells proliferation. Gulla *et al.* designed positively charged AuNPs bearing a tumor targeting peptide (CGKRK) complexed electrostatically with PD-L1siRNA and STAT3 siRNA, with the aim to downregulate immune checkpoint proteins and prevent tumor evasion. Intraperitoneal administration in melanoma-bearing mice revealed selective accumulation of nanoconjugates in tumor tissues, tumor growth inhibition and enhanced overall survivability when compared to untreated animals [57]. Indoleamine 2,3-oxygenase (IDO) is an immune regulatory molecule that inhibits anti-tumor immunity by inducing T cell apoptosis. Zhang *et al.* explored the immunostimulatory effect of gold nanorods (45×15 nm) functionalized simultaneously with gene silencing IDO siRNA and mannose targeting DCs for better internalization of RNA. The nanosystems not only successfully achieved DC-targeted delivery of siRNA but induced efficient DC-specific gene silencing *in vitro* and *in vivo*. The *in vivo* administration to lung carcinoma bearing mice promoted DC maturation and higher antigen-specific T cell proliferation, attenuating tumor growth [58].

Many reports have proven the ability of functionalized AuNPs to enhance antigen presentation while stimulating immune responses for effective vaccination against tumors. Nonetheless, additional studies are required to better understand the complex interaction between tumors, immune system and nanovaccines *in vivo* to eventually translate these promising results into clinical trials. As innate immunity is a key factor in tumor growth, the potential of future immunotherapy can be linked to the development of nanomaterials for the specific modulation of innate immunity cells.

Parasites infections

AuNPs have evoked strong interest in the parasitology and entomology fields, and their bioactivity has been examined on many insect species of economic relevance [59–61]. Among many dangerous parasites, *Plasmodium falciparum* is the main cause of malaria, which remains a health concern in developing countries, responsible for up 283 million cases and 755,000 deaths worldwide. Besides the development of new drugs to prevent and treat the disease, addressing the parasite's sexual stages by means of malaria's transmission-blocking vaccines, represents a pioneering target. Pfs25 is a surface protein identified as target antigen in *P. falciparum* and expressed in zygotes and ookinetes. Therefore, an immune response against Pfs25 could affect the detrimental development of parasites by controlling vector-mediated transmission. Pfs25 is characterized by a complex tertiary structure and four EGF-like repeat motifs, therefore it is

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difficult to obtain a homogeneous product in native conformation. Recently, the expression of codon-harmonized recombinant Pfs25 (CHrPfs25) in an appropriate monomeric conformation has been reported and elicited potent malaria transmission-blocking antibodies in mice [62]. These encouraging results were magnified when CHrPfs25 was covalently immobilized onto different size and shape AuNP surface (spherical, star, cages, and prism) or simpler admixed. CHrPfs25 delivered with various gold nanoparticles elicited strong anti-Pfs25 antibody titers in mice and the antibodies from immunized mice sera showed promising transmission blocking efficacy in mosquito (*An. gambiae*) by means of membrane feeding assays [63].

Non-infectious diseases

With vaccines keeping at bay many infectious diseases, chronic autoimmune diseases have become a health concern of modern times. Autoimmunity arises from an abnormal immune response to autologous proteins which leads to the activation of B and T cells against normal tissues. Currently, the treatment of autoimmune disorders relies on the use of immunosuppressive drugs which compromises the entire immune system, increasing side effects and systemic toxicities. As opposed to traditional treatments, immunotherapy approaches aim to reestablish a normal T cell response by inducing regulatory T cells (T_{regs}). Nanoparticles carrying a disease-relevant autoantigen in combination with immunomodulatory molecules can promote antigen specific T_{regs} and therefore suppress autoimmunity. Unfortunately, only a few disease-specific antigens are known such as myelin in multiple sclerosis (MS), insulin in Type 1 diabetes (T1D), and collagen in rheumatoid arthritis (RA) [9]. The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor inducing tolerogenic DCs that promote the differentiation of T_{regs}. Moreover, AhR activation limits the ability of DCs to promote the differentiation of pathogenic T cells as already proved for MS application [64]. This approach was powerful for T1D: AuNPs were loaded with an endogenous AhR ligand, 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) and proinsulin, a β cell antigen targeted by autoimmune T cells. Administration of the NPs to non-obese diabetic (NOD) mice suppressed the development of the pathology and induced a SOCS2-dependent tolerogenic DC phenotype characterized by the inhibition of nuclear factor κB signaling, a decreased ability to active T_{eff} cells and an increased differentiation of $Foxp3^+$ T_{regs} cells. However, the ability of this approach to ameliorate T1D symptoms was not statistically significant [65]. In a similar manner, proinsulin autoantigen (PIC19-A3) was covalently attached to ultrasmall AuNPs, previously coated with carbohydrates (glucose or mannose and glutathione). Intradermal administration into *ex vivo* human skin using a minimally invasive microneedle proved their ability to diffuse to APC-rich epidermal layer. *In vitro* studies showed that AuNP-peptide complexes impaired maturation of DCs into reactive T cells, promoting the generation of T_{regs}: clinical studies are underway to determine if this system can indeed act as a platform for antigen specific tolerance induction [66].

Conclusions

Development of prophylactic and therapeutic vaccines using nanomaterials constitutes an emerging and promising research field. Recent literature demonstrates that AuNPs, with their unique properties, represent ideal platforms towards a new era of vaccinology. Their high surface area and straightforward functionalization allow simultaneous and multivalent antigen presentation and make them excellent candidates for innovative nano-constructs in the field of vaccine development. AuNPs are readily endocytosed by APCs, affecting increased immunological responses while reducing antigen dosage. Therefore, we expect that some of these nano-formulations will be translated into clinical practice, but this can only be achieved if major challenges are addressed. First, a high level of consistency in the large-scale production, characterization, and reproducibility of engineered AuNPs must be ensured. A better comprehension of the nanoparticle interaction with the immune system and their *in vivo* biodistribution is pivotal to accelerate the rational design of more encouraging nanoformulations. To correctly face this issue, it is fundamental to consider the potential formation of bio-corona around AuNP surfaces. A careful evaluation of such bio-corona formation and the development of protocols or strategy to avoid such phenomenon must be considered in the future nanovaccine developments. Another critical point is the lack of representative animal models which justifies the differences from very promising results from animal tests to poorly encouraging outcomes in preliminary human trials. Finally, the fate of the nanoparticles and their biodegradability must be carefully evaluated, making nanotoxicology a growing research field in which we need to focus our attention.

Conflict of interest

None declared.

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