Exploring the modulation of immune response and oxidative stress of intracellular pathogens using nanoparticles encapsulating drugs

Ramendra Pati Pandey\textsuperscript{a}, Anjali Priyadarshini\textsuperscript{a}, Archana Gupta\textsuperscript{a}, Arpana Vibhut\textsuperscript{a}, Elcio Leaf\textsuperscript{e}, Utpal Sengupta\textsuperscript{b}, Vishwa Mohan Katoch\textsuperscript{c}, Pawan Sharma\textsuperscript{d}, Catrin E Moore\textsuperscript{f}, V. Samuel Raj\textsuperscript{a}

\textsuperscript{a}Centre for Drug Design Discovery and Development (C4D), SRM University, Delhi-NCR, Rajiv Gandhi Education City, Sonipat - 131 029, Haryana, India; \texttt{ramendra.pandey@gmail.com} (R.P.P.); \texttt{anjali.p@srmuniversity.ac.in} (A.P.); \texttt{archana.g@srmuniversity.ac.in} (A.G.); \texttt{arpana.v@srmuniversity.ac.in} (A.V.); \texttt{directorcd4@srmuniversity.ac.in} (V.S.R.); \textsuperscript{b}Stanley Browne Research Laboratory, The Leprosy Mission, Nand Nagari, Sahadra, New Delhi, 110093; \texttt{usengupta2002@yahoo.com} (U.G.); \textsuperscript{c}Rajasthan University of Health Sciences (RUHS), Jaipur, President, JIPMER, Puducherry; \texttt{vishwamohankatoch18@gmail.com} (V.K.M.); \textsuperscript{d}ICGEB (International Centre For Genetic Engineering And Biotechnology), New Delhi 110067, India; \texttt{pawan37@gmail.com} (P.S.); \textsuperscript{e}Institute of Biological Sciences, Federal University of Para, Para 66075-000, Brazil; \texttt{elcioleal@gmail.com} (É.L.)
\textsuperscript{1}Nuffield Department of Medicine, University of Oxford, Big Data Institute, Li Ka Shing Centre for Health Information and Discovery Old Road Campus, Headington, Oxford, OX3 7LF, United Kingdom, \texttt{catrin.moore@ndm.ox.ac.uk} (C.M.)
**Abstract:**

The immune system is a dynamic network of cells and cytokines are the major mediators of immune responses which combat pathogens. Based on the cytokine production, effector T cells differentiate into subsets known as Th1, Th2, Th17 or Treg (T regulatory). This system serves as a barrier to intracellular pathogens, bacterial infections and stimulates the production of reactive oxygen species (ROS), reactive nitrogen intermediates (RNI) and nitric oxide (NO), which diffuses across membranes and engulfs intracellular pathogens. Oxidative stress occurs when ROS, reactive nitrogen species (RNS) production and antioxidant defences become imbalanced. Oxidative stress generated by infected cells produces a substantial amount of free radicals which enables killing of intracellular pathogens. Intracellular pathogens are exposed to endogenous ROS as part of normal aerobic respiration, also aexogenous ROS and RNS are generated by the host immune system in response to infection. Nanoparticles which are designed for drug delivery are capable of trapping the desired drug in the particles which protects the drug from enzymatic degradation in a biological system. The small (subcellular) size of nanoparticles enables higher intracellular uptake of the drug which results in the reduction of the concentration of free drugs reducing their toxic effect. Research on the modulation of immune response and oxidative stress using nanoparticles used to encapsulate drugs has yet to be explored fully. In this review we illustrate the immune activation and generation of oxidative stress properties which are mediated by nanoparticle encapsulated drug delivery systems which can make the therapy more effective in case of diseases caused by intracellular pathogens.

**Key words:** Immune system, Oxidative stress, Nanoparticles, Intracellular Pathogens.

**Introduction:**

The immune system is constantly in a flux, it encompasses a dynamic network of cells, tissues and organs within a host that work in a coordinated manner to defend the body against attacks by "foreign" invaders while also protecting against disease by recognising both "self" and "non-self". Antigens, usually a toxin or foreign substance recognised by the host, are recognized by specialized cells which facilitate their initial destruction followed by elimination from the host. Any microorganism able to cause disease in a host organism can be termed a pathogen. When a pathogen (for example a bacterium, virus or protozoal parasite) infects the human body, after which an internal battle ensues between the host’s innate and adaptive immune system and the pathogen’s assorted virulence mechanisms, together with factors which are able to overcome the immune attack and establish disease. Detection of antigens by the host is complicated as pathogens evolve rapidly; are able to adapt quickly and escape the
immune surveillance which allows the pathogens to infect their hosts and cause disease (Christensen & Thomsen, 2009). Pathogens can be extracellular and intracellular and the mechanism to counter their attack by immune system is varied. As intracellular pathogens reside within the host cell, their elimination and clearing is more complex, the cell-mediated immune response plays a vital role in the host defence against intracellular pathogens such as those causing tuberculosis and leishmaniasis (Urdahl, 2014).

The immune response can be both innate and adaptive; the innate immune response is the first line or primary defence immediately stimulated upon infection. This first line of defence initiated by the host upon the entry of microorganisms to the body involves responses by phagocytic cells that fight pathogens in a nonspecific manner. Antigen presenting cells (APCs) such as macrophages and dendritic cells (DCs), which are spread extensively throughout the body, swallow and process potential microbial antigens via phagocytosis, antigen presentation and activation of T and B lymphocytes to generate an adaptive immune response (Heit et al., 2008). These activated cells cooperate with activated macrophages within the host to abolish intra and extra-cellular pathogens (Storni et al, 2005). Antigens are collected by APCs after which they migrate to the draining lymph nodes with maturation signified by enhanced presentation of antigenic material to major histocompatibility complex (MHC) class I and and/or class II receptor molecules that are subsequently presented to the immune system for development of the acquired immune response. The presentation of antigen alone is not adequate to cause naive T cells to mature into effector T cells. Upon receiving signal, APCs can initiate key T cell responses to the antigen, such as cell survival, differentiation of naive T cells to develop into effector T cells and cytokine secretion (Rescigno et al, 1999; Lima et al, 2004; Reed et al, 2008). CD4+ T cells identify antigens that have been processed by APCs, DCs, macrophages and B cells that express MHC class II molecules.

NPs exert their antimicrobial effects through membrane protein damage, superoxide radical production, reactive oxygen species formation, protein disruption of microbes, damage to proton efflux pump & disruption of electron transport chains and the
generation of ions that interfere with the cell granules leading to the formation of condensed particles (Figure 1).

FIGURE 1. Antimicrobial activities exhibited by nanoparticles include damage of the proton pump, prevention of biofilm formation, oxidation of cellular components, disruption of the electron transport chain & cell membrane of microbes.

**Immune evasion:**

Many pathogens which have the ability to cause acute infections are often cleared effectively by the host's immune system. However, some pathogens which invade the host's cells become intracellular pathogens, and are able to establish persistent and sometimes lifelong infections. Several of these intracellular pathogens manage to evade the host immune system causing disease by replicating inside the host cells. Some bacteria, such as *M. tuberculosis* are able to disrupt the phagosome-lysosome fusion using the PtpA tyrosine phosphatase which preventing the acidification of the
phagosome (Bach et al., 2008). Other mechanisms use the phagosome to create a suitable microenvironment for proliferation: *Legionella pneumophila* safeguards itself from the hosts innate immunity by creating a vacuolar environment which is lacking MHC class II molecules (Clemens and Horwitz, 1992), whereas *C. burnetii* needs an acidic environment for growth and virulence which eliminates the other pathogens (Maurin et al., 1992). Some bacteria can enter a hardy, non-replicating state, termed dormancy for self protection (Rittershaus et al., 2013) *M. tuberculosi*s undergoes a period of reduced cellular growth which maintains a basal metabolism called cellular quiescence (Betts et al., 2002) and true dormancy, a metabolically-arrested spore state promoting survival under adverse conditions, as exhibited by the *Clostridium* spp. (Rittershaus et al., 2013).

Endoparasites are parasites that live in the tissues and organs of their hosts, they encompass both parasites and helminths. Protozoa avoid contact with human immune cells by living in immune privileged sites, *Plasmodium falciparum*, which causes malaria in humans, matures in liver (Patarroyo et al., 2011). The parasite can then travel in the peripheral blood to infect erythrocytes, they target these cells as they lack MHC I receptors, and are not recognised by cytotoxic immune cells (Gomes et al., 2016). *Trypanosoma brucei* is able to invade the central nervous system causing African sleeping disease, the parasite proliferates in blood and lymph and is not recognised by the immune system (Masocha et al., 2007). In addition to evading the immune systems, genetic polymorphisms within the parasites also play a role in immune evasion by a number of protozoans. Two examples are i) *P. falciparum* which has multiple stages during it’s lifetime, their surface antigens are altered after every stage, and ii) *T. brucei* survives through remodeling it’s subsurface protein, this protein is involved in signaling transitions during developmental stages of dormancy and disease progression (Batram et al., 2014). These polymorphic modifications reduce the ability of B cells to make highly specific antibodies against the parasitic antigens (Zambrano-Villa et al., 2002).

*T. brucei* uses its “vector host” to its advantage, the saliva of the Tsetse fly is transmitted along with the parasite, the saliva contains a Gloss2 peptide which suppresses human host release of cytokines TNF-α, IFN-γ, IL-6, and IL-10.
Helminths are able to survive in humans for many years due to their ability to secrete immunomodulatory products, including proteases, protease inhibitors, venom allergen homologues, glycolytic enzymes and lectins (Hewitson et al., 2009). As central components of the “respiratory burst” in activated macrophages and neutrophils, the reactive oxygen species (ROS) and reactive nitrogen species (RNS) cause an oxidative burst which plays an essential role in the host immune defenses against pathogens (Grant and Hung, 2013, Belkaid and Hand, 2014) which can be harnessed to tackle drug resistant infections. Apart from intracellular pathogens there are various extracellular ones which use different modes to evade the hosts immune defence. The Gram positive organism, Staphylococcus aureus (S. aureus) is one of the most important human bacterial pathogens, with worldwide distribution, is a commensal organism which also causes infection in almost any human tissue. Effective treatment of staphylococcal infections has been hampered by the emergence of antibiotic resistance (Spaans, 2015).

**Oxidative Stress:**

Superoxide radicals (O$_2$$^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals (•OH), and singlet oxygen (¹O$_2$) are molecules commonly defined as ROS. They are generated as metabolic by-products by biological systems (Sato et al., 2013; Navarro-Yepes et al., 2014). A proper ROS production forms the basis of other processes such as protein phosphorylation, activation of several transcriptional factors, apoptosis, immunity and differentiation (Rajendran et al., 2014). Redox regulation via ROS/RNS and the antioxidant defences represents a tightly controlled system that can have both deleterious and beneficial effects within the cellular environment. ROS can be generated both intrinsically or extrinsically within the cell. Molecular oxygen generates O$_2$ $^-$, the primary ROS via one-electron reduction catalyzed by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Further reduction of oxygen may lead to H$_2$O$_2$ or OH$^-$ via dismutation and metal-catalyzed Fenton reaction, respectively (Vallyathan and Shi, 2000). Free radicals occur as essential byproducts of mitochondrial respiration and transitionmetal ion-catalyzed Fenton-type reactions (Vallyathan and Shi, 1997).
Inflammatory phagocytes such as neutrophils and macrophages induce oxidative outburst as a defense mechanism towards environmental pollutants, tumor cells and microbes, this results in the generation of both non-specific and specific immune responses. A variety of NP including metal oxide particles induce ROS as one of the principal mechanisms of cytotoxicity (Risom et al., 2005). Nanoparticles have been reported to influence intracellular calcium concentrations, activating transcription factors, and modulating cytokine production via the generation of free radicals (Li et al., 2010) thus contributing towards oxidative stress.

Glutathione-S-transferase (GST) are a family of Phase II detoxification enzymes catalyzing the detoxification of electrophilic compounds, it protects cells from mutagens and carcinogens as a free radical scavenger along with glutathione (Hemachand, 2002). Glutathione removes H$_2$O$_2$ in the cytosol (Reed,1969). H$_2$O$_2$ is formed near the membrane during phagocytosis, and it can simply diffuse into the cytosol. In the cytosol, glutathione reacts with H$_2$O$_2$ through a chemical reaction catalyzed by glutathione peroxidase which results in glutathione disulfide (GSSG) (Paul, 1970). Glutathione reductase (GR) catalyzes the regeneration of glutathione from GSSG, utilizing NADPH generated by the hexose monophosphate shunt (HMPS).

Glutathione Peroxidase (GPx) isoenzymes use GSH as a donor of reducing equivalents to detoxify H$_2$O$_2$, in various organic compounds (Briviba, 1998). Mitochondria in normal mammalian cells are the principal endogenous sources of ROS, the main exogenous sources of ROS are drugs and other xenobiotics. Cells have an elegant defense system against ROS, consisting of antioxidant enzymes and low molecular weight substances capable of scavenging many different ROS. Glutathione Peroxidase (GPx) has a crucial role in the defence mechanisms against damage by catalyzing the reduction of H$_2$O$_2$ and a large variety of hydroperoxides into water and alcohols (Sies, 1995). The cellular pool of glutathione is replenished by two mechanisms: glutathione regeneration from GSSG mediated by GR and de novo glutathione synthesis. By regenerating glutathione from GSSG, GR facilitates cytosolic H$_2$O$_2$ detoxification, which protects phagocytes from oxidative damage and
sustains oxidative burst-mediated bactericidal activities (Reed, 1969). In oxidative stress there is either an excessive production of ROS or a significant decrease or lack of antioxidant defense. The removal of H₂O₂ or other hydroperoxides by GPx requires GSH as cofactor. GSH is a tripeptide of glutamate, cysteine, and glycine, which is found ubiquitously in eukaryotic cells at a concentration between 1 and 10 mM. GSH has a potent electron-donating capacity. Its high redox potential reduces GSH which is both a potent antioxidant and a convenient cofactor for enzymatic reaction.

These molecules are all associated with the regulation of apoptosis (Riley et al., 2006). NO is a component of the innate immune system, and is involved in both the pathogenesis and control of several types of viral, bacterial and parasitic infections (Bogdan, 2001). Furthermore, NO is able to modulate the immune response via the regulation of apoptosis and the upregulation of cytokine mRNA expression (Hanum et al., 2003). The regulation of NO production in tuberculosis appears to be very complex, due to the ability of various mycobacterial cell wall components to stimulate the release of NO (Underhill, 1999). Basu et al reported that M. tuberculosis secretory proteins control cell signaling, ROS and MTSA-10 are also important in the modulation of macrophage immune functions (Basu et al., 2006, 2009). Secretory proteins of M. tuberculosis induce a TH1 bias in T cell response (Pandey et al., 2011).

Mycobacterium abscessus is a non-tuberculous mycobacteria (NTM) which represents a pathogen causing a substantive but often underappreciated burden of disease worldwide. Although causing pulmonary diseases that resembles tuberculosis (TB), M. abscessus does not respond to standard anti-TB therapy due to its natural resistance to most antibiotics.

The integrated dynamics between oxidative stress and the immune cell function of an intracellular pathogen that can sense and acclimatize to the continuously changing host environment during infection. Both oxidative and reductive stresses induce redox cascades that have the potential to alter pathogens signal transduction, DNA and RNA synthesis, protein synthesis and drug resistance (Manke et al., 2013). Maintaining a suitable redox balance is necessary for a good clinical outcome, many prodrugs are only effective upon bio reductive activation. Proper homeostasis of oxido-reductive
systems are essential for pathogens survival, persistence and subsequent reactivation. As in humans, the exposure of bacteria to ROS causes damage to a variety of macromolecules, resulting in mutations and often in cell death thus contributing to the generation of a host defence. ROS function as signaling molecules that lead to a coordinated response in bacteria under redox-stress conditions. Metallic nanoparticles (NPs) offer a novel potential means of fighting bacteria because they exert their effects through membrane protein damage, superoxide radicals and the generation of ions which may cause oxidative stress.

**Role of Nanoparticles in specific immune response against intracellular pathogens:**

Microorganisms in infected tissues can become resistant to antimicrobials through a number of routes, these may include, amongst others, protection by biological structures around the infection foci. The bacteria will secrete glycocalyx through during adhesion to the host cells which can lead to increased protection and provide an increased resistance to antibacterial agents (Bakker, 2002), eradication of the intracellular pathogen will not occur (Allen, 1998). Research has concentrated in this area, loading antibiotics into the colloidal carriers, liposomes and nanoparticles, has shown an improved drug delivery to infected cells (Allen and Martin, 2004). Currently there are two types of delivery systems: liposomes and nanoparticles. Intracellular drug delivery systems need to be biocompatible with the host and should minimize nonspecific cytotoxic effects to healthy tissues (Seale-Goldsmith, 2009). Nanoparticles have been produced using a variety of materials, including poly(lactide-co-glycolide) (PLGA), poly-lactic acid (PLA), polymethacrylic acid (PMA), polyethylene glycol (PEG), natural polymers such as chitosan, gelatin, or alginate. Nanomaterials have physicochemical properties which are different from other substances. The possible routes by which nanomaterials migrate in the human body include the following:

1) Endocytosis: Nanomaterials enter the cells when they are surrounded by the cell membrane but don’t pass through it.
2) Penetrates the cell membrane using hydrophobic particles.
3) Nanoparticles <5 nm are transported across the cell membrane channel. The nanoparticle structures allow a better retention of the drug inside the polymeric network and can slowly be degraded by esterase action.

There are two types of nanoparticles:

a. Nanospheres (these have a solid framework)

b. Nanocapsules (these have a liquid central cavity surrounded by a wall).

Nanoparticles are classically prepared by the emulsion polymerization of alkylcyanoacrylic monomers in the presence of a specific drug. This reaction is induced by a nucleophilic agent. In some cases, the antimicrobial agent may itself induce the anionic polymerization of the monomer. The linkage of the drug to polymer by covalent bond may result in its non availability which is not desirable as a therapeutic drug. Therefore, the antimicrobial activity of the nanoparticle drug formulation must always be compared in vitro with the free drug as the control to understand the bioavailability. The release of the drug from the nanoparticles is dependent on the degradation rate of the nanoparticle. The release can be maximised using enzymes for its degradation. The drug may be used to form the nanoparticle through encapsulation within a polymer matrix, or it can through attachment to the surface of a solid nanoparticle which acts as a carrier. The incorporation of surface targeting components helps to localize to the affected tissue which may improve the therapeutic efficacy, along with the presence of molecules to enhance the cellular penetration such as cell-penetrating peptides (CPPs). CPPs are short, cationic peptides, typically derived from the HIV TAT proteins (Fonseca, 2009), which readily translocate through cell membranes. The addition of CPPs, such as TAT, to the surface of a nanoparticle can increase the efficiency of intracellular delivery of the nanoparticles (Juliano, 2009; Schmidt, 2010). The CPP probably promotes direct translocation through the membrane via electrostatic interactions or the it binds to specific receptors on the membrane and induces rapid receptor-mediated endocytosis (Schmidt, 2010) which promotes cellular penetration and as a result helps combat infection by intracellular pathogens. Cellular internalization of NP activates immune cells including macrophages and neutrophils, contributing to reactive oxygen species and reactive nitrogen species (ROS/RNS). Based on these findings, it is postulated
that the oxidative stress and inflammatory responses may induce the occurrence of immunotoxicity directly or indirectly. Cytokines are soluble signaling molecules used for intracellular communication. Upon such recognition, activation of CD4+ T cells and T helper (Th) cells, differentiation can take place into Th1 and Th2 type subsets (Seder and Hill, 2000). These two subsets secrete a variety of cytokines. A typical Th1 type cytokine is IFN-γ which induces phagocyte-mediated defense against infection, particularly intracellular microbes. In contrast, Th2 type cytokines such as IL-4 and IL-10, down regulate Th1 type immune responses and activate B cells to stimulate antibody synthesis (Lima et al, 2004).

**Nanoparticles against pathogen:**

Nanomedicine is an application of nanomaterials that provides solutions to current unmet medical needs. Recent advances includes the development of innovative nanoparticles to target reactive oxygen species (ROS) (Figure 2). Oxidative stress has been known to contribute a great deal in altering the bacterial membrane permeability resulting in damage of cell membranes. There are various examples of metallic NPs regarding this aspect such as nano silver ions activate oxygen and produce reactive oxygen ions and hydroxyl radicals, which can hinder bacterial proliferation or destroy bacterial cells.

Metallic nanoparticles (NPs) offer a novel potential means of fighting bacterial infections and combating drug resistant infections. Nanoparticle activities can also target multiple biomolecules and have the potential to reduce or eliminate the evolution of multiple drug resistant organisms (MDROs) (Slavin et al., 2017). Different NPs employ different mechanisms to destroy bacteria. Metallic NPs use several modes of action to destroy bacteria: they penetrate the bacterial cell wall and form pores on the surface of the membrane which, in turn, cause free radical formation which can destroy the cell membrane (Prabhu and Poulose, 2012).

A recent report describes an enhanced activity of ciprofloxacin and gentamicin, among others, with both synergistic and additive effects, when applied in combination with AgNPs (Panáček, 2016). The enhancement effect was higher in antibiotic-
resistant strains than in antibiotic-susceptible strains, because AgNPs affect the cell membrane and cell wall integrity favoring antibiotic action, leading to a “restored” susceptibility for some antibiotic resistant strains (Panáček, 2016). It has been reported that RBx 11,760 loaded nanoparticles have potential to be used as nanomedicine against drug resistant S. aureus infections (Barman, et al., 2018).

**Bio-polymeric nanoparticulate drug delivery system:**

Nanoparticles offer the ability to interact with complex biological functions at the scale of biomolecules, implying enormous opportunities for novel applications in medicine, including diagnostics, medical imaging, targeted delivery and immunotherapy (Zhang, 2015). Nanoparticles are defined as a material with dimensions of 0.1-100 nm that behaves as a whole unit in terms of its transport, properties and unique characteristics.

Polymeric nanoparticles are defined as nanoscale drug delivery platforms assembled by biodegradable polymers, dendrimers, conjugates or micelles (Poilil Surendran et al., 2017). The nanoparticles can easily be manipulated through the particle size and surface characteristics. Such modifications increase drug therapeutic efficacy and reduce the side effects. Polymeric biodegradable nanoparticles have been explored for vaccine formulations, in which antigen is encapsulated in polymers such as PLGA, polylactide or Chitosan (Pandey et al., 2011). For example, it has been reported that smaller nanoparticles (~25 nm) travel through the lymphatic more readily than the larger particles (~100 nm) and accumulate in lymph node resident dendritic cells (Reddy et al., 2007). Polysaccharide-derived nanoparticles and nanostructured surfaces help to improve biocompatibility of cell toxic material, together with new immobilization approaches, which are currently in development for novel bionanoparticle-derived pharmaceutical formulations. Panda et al., (2009) revealed that microparticles elicit better antibody response than nanoparticles, and improve the immunogenicity of polymer particle entrapped antigen. Admixture of micro-particulate antigen and alum elicit memory antibody response. Biopolymeric nanoparticles like Albumin (Goosen, 1985), Alginate (Wee and Gombotz, 1994), Collagen (Gilbert et al., 1990), Gelatin (Shinde and Erhan, 1992; Safran et al., 1991),
Hyaluronic acid (Illum, 1994), Polysaccharides (Artursson, 1984), Chitosan (Verma, 2011; Artursson, 1984), have attracted considerable attention as potential drug delivery devices in view of their applications in the controlled release of drugs, their ability to target cells / tissues, and in their ability to deliver drugs in optimal dose at targeted sites (Langer, 2000). The synergistic effect of NPs when placed in combination rather than using a single NP and for combinations to target a broad spectrum of both Gram-positive and Gram-negative bacteria, could be useful to healthcare practitioners and biomedical engineers in the development of medical devices that target a wide range of bacterial pathogens (Bankier et al., 2019).

FIGURE 2. Schematic representation of nanoparticles as a carrier system and its applications.

**Conclusion:**

Infectious diseases caused by pathogenic bacteria are one of the most common causes of death worldwide and are a constant health risk in all countries (Marova et al., 2011). Today, the burden of infectious disease on health, economy and other social aspects is so complex, that the worldwide cost cannot be estimated (El-banna et al., 2012). Multi-drug resistant (MDR) pathogens constitute one of the most severe worldwide public health problem. Apart from those, the biodegradable polymers can
be utilized as drug delivery system because of several advantages such as biodegradability, biocompatibility, enhanced circulation and reduced toxicity. The main objective in using the nanoparticles as a delivery system is the control led release of encapsulated controlled drugs in order to accomplish the site specific action at the therapeutically optimal level. In addition, they can also influence the entrapment efficiency, release of the drugs from the nanoparticles and stability of nanoparticles. The area of modulation of immune response and oxidative stress using nanoparticles encapsulating drugs is yet unexplored. In this review we are trying to discuss the possible hypothesis whether the immune activating and generation of oxidative stress properties of nanoparticles encapsulated drugs delivery system can be co-related for progressing toward more effective therapy and immune response in case of infections caused by intracellular pathogens. Using antibacterial encapsulated nanoparticulate systems we may lead to a delay or inhibition of the resistance development. The shining ray of hope amidst the plethora of antimicrobial resistance is that the microbes would require multiple gene mutations in the same bacterial cell to become resistant to NPs. It will be interesting to explore further; an interplay of immune response, ROS and RNS created by intracellular pathogens, which warrant detailed evaluation on the signaling pathways to ascertain the extent of interdependence.

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