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Nanotechnology Based Therapeutics, Drug Delivery Mechanisms and Vaccination approaches for Countering *Mycobacterium avium* subspecies *paratuberculosis* (MAP) Associated Diseases

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ABSTRACT

Johne's Disease (JD) is a contagious fatal granulomatous enteritis, known to affect ruminants and is caused by the acid-fast *Mycobacterium avium* subspecies *paratuberculosis* (MAP). The bacterium has also been linked to Crohn's Disease (CD) in humans. Treatment options are scarce with culling practiced in the case of Johne's Disease (JD) and administration of anti-inflammatory drugs for pain and inflammation in case of CD. In both cases antimicrobial therapy against MAP does not have the ultimate potential. The very promising, yet untapped potential of nanotechnology offers a suitable platform for developing new therapeutic strategies for diseases caused by the bacteria. Uniformity, specificity and reproducibility are some of the characteristics of nanotechnology that can be exploited for the treatment of infectious diseases. Factors like cost, efficacy, safety and bioavailability of drugs can be greatly improved when the drugs are delivered with precision and at a controlled delivery rate to the target location. Nanotechnology can help in achieving these targets. This review discusses the current scenario of available therapeutic approaches and proposes drugs targeting strategies and vaccine development methods for the treatment and prevention of MAP related diseases.

Key words: *Paratuberculosis*, Johne's disease, therapeutics, nanotechnology, drug delivery, vaccination

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP), an acid fast bacterium is the causative agent of Johne's Disease (JD), a contagious bacterial disease known to affect the large intestine of ruminants. The MAP is also known to infect a wide range of non-ruminant species like

pigs, horses, dogs and a wide range of wildlife and birds (Ayele *et al*., 2005). The bacteria characteristically attach itself to the mucosal wall of the ileum followed by infection of the intestinal macrophages leading to thickening of the intestine. Shedding of MAP in milk and faeces is prevalent in clinically and sub-clinically infected animals, though in a lesser extent in the latter (Stabel *et al*., 2014). The shedding of MAP organism through faeces and milk contaminates the environment animals live in. Presence of MAP in milk and its ability to survive pasteurization raises alarm bells as new research findings its zoonotic potential in Crohn's Disease (CD) (Singh *et al*., 2010).

Human exposure to MAP includes co-habitation with domestic animals and exposure by MAP contaminated food (Singh *et al*., 2011). Over the years, compelling evidences suggest MAP to be the causative agent of CD prevalent among humans (Chamberlin *et al*., 2001). First report of a possible link between JD and CD was reported in the 1980's where Chiodini *et al*. (1984) isolated MAP strains MAP strains were isolated from CD patients. Many similarities exist between the two diseases like it infects ileum, thickening of intestinal mucosa and sub-mucosa, formation of granulomas and infiltration of macrophages. However, JD cases do not suffer from abdominal pain, fistulae, constipation and they lack fibrosis, ulceration and skip lesions as seen in CD patients (Selby, 2000). The bacterium has been isolated from gut, milk and blood of Crohn's patients (Naser *et al*., 2000, 2014). Greenstein (2003) support the linkage between MAP and JD by stating that MAP meets Koch's postulate by fulfilling its four criteria's namely; it is found in tissue of CD patients, the bacteria is culturable, it has the ability to replicate the disease when inoculated into other animals and it can be re-isolated from the diseased animals. Besides, MAP has also been reported to be candidature pathogen for diseases like type 1 diabetes (Dow and Sechi, 2011), irritable bowel syndrome (Scanu *et al*., 2007), ulcerative colitis (Pierce, 2010) and sarcoidosis (El-Zaatari *et al*., 1996).

The MAP also poses a significant economic impact on the animal industries, mainly due to losses owing to low milk production, culling of infected animals, low slaughter value and treatment costs (Behr and Collins, 2010). The latest study conducted in 373 dairy farms in Canada revealed the mean loss for an average herd was $2992 annually (Tiwari *et al*., 2008). It is estimated that the diary industry in USA suffers an economic loss of more than $200 million due to JD (Robins *et al*., 2015). A study in India has calculated the approximate economic loss per sheep/farmer/year to be around Rs 1,840 (US$ 38.33) in JD affected sheep (Vinodhkumar *et al*., 2013). In another study the loss estimated in a Holstein Frisian (H/F) dairy farm was around Rs 1,63,800.0 (US$ 2465) in 180 days (Rawat *et al*., 2014).

Globally, treatment of infectious disease is a challenge as microbes become resistant to conventional antibiotics. Hence the focus has shifted to nanotechnology based therapeutics where, the unique physical and chemical properties of nanomaterials are exploited to uplift the pharmacological behaviour, dosage required and targeted as well as efficacious delivery of medicines and drugs. Their small size makes it possible to surpass physiological barriers and characteristics such as high surface area to volume ratio, which may allow for increased interaction with pathogen membranes and cell walls (Blecher *et al*., 2011). Nanotechnology and nanobased therapeutics may entirely change the whole concept of healthcare in coming years (Dhama *et al*., 2008; Chakravarthi and Balaji, 2010; Manuja *et al*., 2012; Num and Useh, 2013; Svenson, 2014). This review discuses the various possible treatment availabilities of MAP associated diseases with special focus on nanotechnology owing to the positive impact this field has on treatment with other infectious diseases.

THERAPEUTIC OPTIONS

Till date, there is no effective cure for CD and treatment options aim at improving the symptoms. Initial treatment involves the administration of steroids to reduce inflammation. Steroid medication ranges from corticosteroids to milder forms like aminosalicylic acid (5-ASA compound) (Hanauer, 1996). Anti-inflammatory drugs such as budesonide and mesalazine (mesalamine) are commonly used in the medical management of patients suffering from mild to moderate CD. A study revealed the effective dosage required for budesonide was 9 mg day^{-1} or higher to initiate the remission in active mild or moderate cases and 6 mg day^{-1} to maintain the remission in CD cases (Moja *et al*., 2015). Over the years treatment of CD has significantly advanced by the administration of anti-Tumor Necrosis Factor (anti-TNF) agents like infliximab, adalimumab, certolizumab pegol and natalizumab. Methotrexate, a dihydrofolate reductase inhibitor is another drug commonly used to induce the remission in CD. A study in 2014 revealed that an intramuscular dose of 15 mg $week^{-1}$ of methotrexate is sufficient to maintain the remission of CD (Patel *et al*., 2014). Most of the time the patients respond to one or a combination of these therapies and if all options fail, surgery is recommended. However, even after surgery the cases of CD are known to relapse (De Cruz *et al*., 2015).

Recently, stem cell therapy has also been used to induce the remission of CD (Martinez-Montiel *et al*., 2014). In a study, 10 patients with CD were administered stem cell therapy with receiving two doses of mesenchymal stromal cells intravenously and this therapy appeared to be safe and feasible for the treatment of CD (Duijvestein *et al*., 2010). However, larger group study needs to be done to confirm its efficacy for the treatment of CD. Recent research has also shown that autologous bone marrow-derived mesenchymal stromal cells have been able to cure fistulae that resulted due to CD (Ciccocioppo *et al*., 2015). Treatment by Fecal Microbiota Transplantation (FMT) method has been known to cure many intestinal diseases like *Clostridium difficile* infection and in certain cases of Inflammatory Bowel Disease (IBD) (Angelberger *et al*., 2013). A pilot study was conducted that consisted of the largest known sample of refractory CD which underwent FMT (Cui *et al*., 2015). Laboratory prepared fresh fecal microbiota suspension was administered to patients midgut through a tube. The results highlighted a convenient method for treatment of CD that is safe, feasible and efficient. All these approaches aim to reduce the symptoms associated with CD like pain and diarrhoea do not cure CD.

In case of JD, culling of the diseased animal is the standard practice for the management and control of the spread of the disease within herds or flocks. However, in practice culling has practical problems in most of the countries due to huge economic losses (Tamba *et al*., 2014). As it is not easy to pay the farmers for all culled animals, since incidence of the disease is very high in developing and poor countries. Moreover, detection of *paratuberculosis* is not possible with present days diagnostic tools, therefore, culling practice cannot be relied as sole method for controlling paratuberculosis. The MAP is also transmitted vertically (from parents to offsprings through semen, during pregnancy, etc. (Robins *et al*., 2015). Due to vertical transmission of disease, culling has very limited scope in control of this disease (Sohal *et al*., 2015). So, far anti-mycobacterial treatment has not been applied in animals for treating MAP infection because of fear of entry of drugs into food chain by milk and meat. Therefore, safe alternatives are needed to be developed that can not only cure JD in animals but also are safe for the public health.

ANTIMICROBIAL OPTIONS FOR MAP WITH SPECIAL REFERENCE TO NANOBASED TECHNIQUES

The MAP is resistant to standard anti-tuberculous drugs and is known to be difficult to eradicate *in vivo* infections (Gangadharam *et al*., 1987). However, some antibiotics like rifabutin, clarithromycin and azithromycin are said to have enhanced activity against MAP and it has shown to work better in combinational therapy for both JD and CD (Panteix *et al*., 1993). In a study published by Chiodini *et al*. (1993), BALB/c mice were infected with MAP and then rifabutin was administered as treatment. The results showed that mice treated with 50 mg of rifabutin per kg found a reduction in bacterial count in the liver and spleen over a 6 month period. The findings revealed that rifabutin could be considered as a suitable chemotherapeutic drug for long-term treatment of MAP infection and should be further investigated in multidrug regimens (Chiodini *et al*., 1993). In another study, 46 CD patients were treated with rifabutin in combination with clarithromycin or azithromycin. The analysis showed that in combination these drugs show clinical improvement in CD (Gui *t al*., 1997). In addition, drugs such as amikacin, ciprofloxacin, levofloxacin, rifampicin posses varying degrees of susceptibility towards MAP (Krishnan *et al*., 2009). Greenstein *et al*. (2007) showed that the accepted use of methotrexate and 6-mercaptopurine (6-MP) for therapy of IBD may be due to the inhibitory action of the drugs on MAP, instead of the generally prevailing idea that the primary mechanism of action is the drug's ability to decrease the production of pro-inflammatory cytokines. Nazareth *et al*. (2015) investigated the growth of MAP in macrophages of CD patients under the influences of Infliximab treatment. A lower MAP CFU count was reported in macrophage cultures.

The MAP being an intracellular bacterium relies on iron for its survival and replication. Scientists have taken advantage of this property by using gallium (Ga), a semimetal that function as an iron mimic. At the site of inflammation, phagocytes take up Ga which then interferes with the iron-dependent cellular pathways leading to the death of the bacteria (Olakanmi *et al*., 2000). *In vitro* antimicrobial activity of Ga has been reported against various bacteria including MAP. Gallium nitrate (GaN) treatment of calves infected with MAP resulted in decreased colonization of MAP in tissue (Fecteau *et al*., 2011). Recently, Fecteau *et al*. (2014) compared the antimicrobial activities of gallium maltolate (GaM) with GaN against MAP. The findings demonstrated that GaM had higher antimicrobial activity against MAP than GaN and it was hypothesized that this higher efficiency was due to GaM being more lipid soluble than GaN (Fecteau *et al*., 2014).

Mohanty *et al*. (2013) studied the effect of silver nanoparticles on different mycobacteria strains. The results revealed the antimicrobial effect of silver nanoaparticles was higher for *Mycobacterium smegmatis* when compared to *M. marinum*. This strain selective mycobactericidal activity showed no DNA damage, cytotoxic or toxic effect to macrophages upto treatment with 5 ppm of silver nanoparticles making silver nanoparticles a suitable therapeutic option for mycobacterial infections. Study of Singh *et al*. (2015) further backed anti-mycobacterial efficiency of silver nanoparticles. This study revealed that the silver nanoparticles were specific towards mycobacteria strains when in comparison to other Gram positive and Gram negative bacteria. These studies can pave the way for future research where the use of silver nanoparticles as a potential therapeutic approach for MAP infections in animals and humans would be evaluated.

Presently anti-MAP treatment for JD is not recommended and not been the focus of major studies as the benefits outweighs the cost and hence not preferred. Moreover, drugs may enter into food chain through meat and milk. In the absence of control measures the prevalence of the disease has been increased over the years (Singh *et al*., 2014). Therefore, a treatment option of JD is required that is cheap and safe in terms of food safety for humans.

NANOTECHNOLOGY BASED DRUG DELIVERY APPROACHES FOR MAP

Drug delivery system is the method or process of administering a pharmaceutical compound to achieve a therapeutic effect in humans or animals (Tiwari *et al*., 2012). To reach the highest level of efficacy of an administered drug, two factors need to be considered, namely; the ability of the drug to present itself to the body at a specific required rate and to direct the active entity of the drug specifically to the site of action thereby increasing the action of the drug and reducing the side effects (Wikberg *et al*., 1997). It is generally accepted concept that the efficacy of the drug is enhanced by the delivery of the active substance at the site of infection (Goracinova *et al*., 2012). Normally, drugs follow the standard process of absorption, distribution, metabolism and elimination after administration. These factors determine the concentration, retention and the site of action of the drug administered. A favourable therapeutic response is initiated when the sufficient amount of the drug is available at the site of action for the active compound to work. The inability of anti-MAP therapy to effectively work *in vivo* is probably due to the failure of the drugs to target specific sites of infection. *Paratuberculosis* is an intracellular organism and therefore, drugs may reach with difficulty to intracellular locations for targeting MAP (Czyz *et al*., 2014).

In the case of JD and CD, the mechanism of action of drugs should be directed towards the gastrointestinal region, namely the terminal ileum and the large intestine where the infection is known to take place. Drug delivery into the gastrointestinal track faces several hindrances like poor stability, low bioavailability and low solubility. These problems can be overcomed by the development of nanoparticle based delivery systems. Nanomaterials can be used to encapsulate and protect the drugs to increase their efficacy and specificity. Nanoparticle surfaces can also be modified to increase bioadhesion to target cells (Ensign *et al*., 2012). Bellaire and Narasimhan (2013) showed that antibiotic (amikacin) encapsulated in polyanhydride nanoparticles exhibit stronger anti-MAP effect than soluble form of antibiotic. It has been shown that nanomaterials can be used to target phagocytic cells infected with mycobacteria (Mathuria, 2009). Therefore, it can be proposed that antimicrobial therapy for MAP can be linked with a drug delivery system to target the ileum and the large intestine for the treatment of JD or CD.

The pH variations along the gastrointestinal tract can be exploited in order to control the release of drug at target regions, protect the drugs from extreme conditions and enhance their intestinal absorption (Wang and Zhang, 2012). This system can be divided into two basic types: polymers that undergo solubility changes in accordance with pH variation and polymers that contains pH sensitive bonds, which release the drug molecule when these bonds are cleaved based on the change in pH (Mura *et al*., 2013). Wikberg *et al*. (1997) designed a pH dependent drug delivery system. In this system pH variations in the intestinal compartments were taken into consideration. Their system consisted of coating the drug polymeric film with pH dependent solubility. Polymers such as hydroxypropyl methyl cellulose phthalate (HPMCP 55) and co-polymers of methacrylic acid and ethyl acrylate are soluble above pH 5.5 will dissolve immediately after passage through the stomach. The pH dependent dissolution is another approach that can be considered as drug delivery system where the outer coating of the drug will dissolve at a particular pH. Polymers like polyethylacrylate or polymethacrylate (PEA/PMA) can be used in such cases. Commercially available PMA copolymer (Eudragit® S 100-Evonik Industries) is available for drug release in the ileal region of the gastrointestinal track (Dai *et al*., 2004). So, coating anti-MAP drugs with Eudragit® S 100 for release into ilea region can be a good option for treatment of JD.

Nanoparticles formulated from biodegradable polymers have been used to improve the bioavailability of drugs. Sarmento *et al.* (2007) showed that oral administration of insulin coated with dextran sulphate/chitosan nano-particles improved the bio-availability. Chitosan has muco-adhesive and absorption enhancement properties and dextran sulphate will give protection in low pH environment ensuring the better intestinal absorption of the drug. The pH of ileum is round 7.5 hence, polymers used as a coating need to dissolve at round 7.5 and at the same time, a time lag would need to be considered if the drug would need to pass through the various parts of the GI track before reaching the ileum. Chitosan nanoparticles can offer an excellent mechanism for the delivery of drug to ileum region. Chitosan nano-particles maintain their stability through the GI track due to electrostatic interactions. However, on reaching at pH 7.5 the chitosan becomes unstable due to the deprotanation and ultimately collapses, releasing the drug to the target site i.e., ileum (Lin *et al*., 2009). Therefore, anti-MAP therapy can be designed wherein the anti-MAP drug can be loaded on chitosan nanoparticles for specific delivery of drug to the target site of infection.

Some nanoparticles swell in pH dependent manner and this property can be exploited for drug delivery (Peppas, 2004). Polymers like Polymethacrylic Acid (PMAA) attached with polyethylene glycol (PEG) exhibit reversible, pH-dependent swelling behaviour due to the arrangement of inter-polymer complexes between protonated pendant acid groups and the etheric groups on the graft chains. In the stomach region the PMAA nano-particles held a hydrophobic collapsed state as a result of protonation of carboxyly groups (Colombo *et al*., 2009). After the gastric passage the increase in pH to 7.4 caused the nano-particles to swell due to carboxyl ionization and hydrogen bond breakage. When insulin was loaded with onto such a system, it was reported that at a pH of 7.4, 90% of insulin were released (Peppas, 2004). Hence, a similar treatment can be conceptualized for anti-MAP drug delivery system. Anti-MAP antibiotics can be loaded on nano-particles such as chitosan nano-particles, PMAA grafted with PEG for the delivery into the ileum region of the gastrointestinal track for treatment of JD and CD cases.

Another approach would be a system that comprises of a layer-by-layer milk protein casein coated iron oxide nanoparticles (CN) construct that can be loaded with anti-MAP drug for its delivery into the ileal region of the intestine. This approach has been tried by Huang *et al*. (2015) where doxorubicin (DOX) and indocyanine green (ICG) were selected as model drugs. The coating was stable under the acidic gastric conditions and then the casein outer layer was degraded by the intestinal proteases releasing the loaded drugs. The localization of the drug in the small intestine was confirmed in mice by *in vivo* imaging after passage through the stomach without significant degradation of the drug (Huang *et al*., 2015).

Recently, a lipid nanocarrier containing phage lipolytic enzyme has been designed as drug delivery system for treating mycobacterial infection (Pereira *et al*., 2015). Pereira *et al*. (2015) loaded LysB enzyme (a phage mycolylarabinogalactan esterase that break the lipids in cell wall of mycobacteria) on lipid nanocarrier. This system selectively targets the phagocytic cells facilitating intracellular accumulation of LysB causing mycobacterial suppression. Studies revealed that the designed system was able to inhibit the growth of *M. smegmatisthan* free LysB owing to the facilitation of internalization by the nanocarrier. Such mechanism can also be designed for MAP infection. Table 1 summarizes the various proposed mechanism of orally administered delivery systems for anti-MAP specific drugs.

Table 1: Proposed mechanisms of orally administered delivery systems for anti-MAP drugs using nanotechnology

Drug delivery system	Drug delivery/drug type	Mechanism	Target	References
pH dependent solubility	Drug coated with polymers that show pH-dependent dissolution	Eudragit® S100 dissolves at ph >7 Coating of drug with HPMCP	Ileal region of intestine	Eudragit® evonik industries Wikberg *et al*. (1997)
pH dependent response	Drug chitosan nanoparticles	Deprotination of chitosan at pH 7.4 causing the collapse of nanoparticles and release of drug	Ileal region of intestine	Lin *et al*. (2009)
pH dependent swelling	PMAA-(PEG) copolymer NP's loaded with drug	High pH of 7.4 will cause swelling of NP's which will cause the release of the drug	Ileal region of intestine	Peppas (2004)
pH dependent and time lag	Chitosan-dextrate sulphate	Deprotonation of chitosan at pH 7.4 and dextran sulfate promotes longer circulation time	Ileal region of intestine	Sarmento *et al*. (2007)
Enzymatic responsive	Casein coated magnetic nanoparticles loaded with drug	Casein stable under acidic gastric condition and then degrade due to the intestinal protease releasing the drug	Ileal region of intestine	Huang *et al*. (2015)
Hydrogels	Antigen responsive hydrogels	Free antigen causes the hydrogel to swell, releasing the drug	Ileal region of intestine	Miyata *et al*. (1999)
Nano-drug	Drug loaded chitosan based magnetic nanocomposites	Enhanced antibacterial activity	MAP bacterium specific	El Zowalaty *et al*. (2015)
Nano-drug	Silver nanoparticles	Metalic nanoparticles kill by permeabilizing the cell membrane of pathogen	MAP bacterium cell wall	Singh *et al*. (2015)
Nano-drug	Phage derived protein delivery system	Degardation of lipids present in cell wall of mycobacterium	MAP bacteroium cell wall	Pereira *et al*. (2015)

NANOTECHNOLOGY BASED APPROACHES IN VACCINE DELIVERY FOR MAP

Vaccination for JD is at present the most reliable preventive and therapeutic approach for controlling the disease (Singh *et al*., 2014). Both live and killed vaccines have been used (Singh *et al*., 2014). Adjuvants are added to the MAP vaccines, which are used as continuous stimulation agents of antigen for immune system (Bastida and Juste, 2011). Our group is preparing vaccine using GERBU adjuvant (Singh *et al*., 2014). The GERBU adjuvant formulation consists of two factors, cationic nanoparticles in a colloidal suspension and immunomodulator derived from *Lactobacillus bulgaricus* cell wall.

Nanotechnology based vaccination has been the focus of interest as a suitable alternative to traditional vaccine preparations. Most of the systems consist of nanocarrier based on the principle that particles smaller than 10 μm are easily taken up by macrophages and Dendritic Cells (DC). It has advantage of improved efficiency in terms of antigen recognition and presentation by facilitating the cellular uptake of antigens (Oyewumi *et al*., 2010). Emulsions, synthetic or natural polymers to carbon based systems have been used as nanocarreirs in vaccination (Kim *et al*., 2014). Coating of these nanocarriers with molecules such as peptides or carbohydrates or lipidswill facilitate higher efficiency in delivery of antigen and adjuvant for the production of specific immune response against vaccines. Liposomes and nanovesicles derived carrier systems have been exploited recently in the field of vaccine development owing to their advantages of plasticity and versatility (Schwendener, 2014). Features such as charge, size, distribution, entrapment can easily be altered in liposomes to achieve the desiredcharacteristics for vaccine development.

Liposomes based MAP vaccines can be proposed using nano-carrier based system wherein, MAP antigens or peptides are entrapped in the inner space of liposomes, where they get intercalated into the lipid bilayer and adjuvants can be attached onto the surface of these liposomes by simple

adsoprtion or chemical linkage to enhance the immune response (Schwendener, 2014). Nanosized Virus like Particles (VLP) can act like adjuvants or facilitate antigen uptake by antigen presenting cells (Aguilar and Rodriguez, 2007). Their small size improves stability and increase efficacy of transport across barriers (Zhao *et al*., 2014).

Dendritic Cell (DC) mediated immune response is now considered a good vaccine model for MAP related infectious diseases. It is based on the principle that the Fibronectin Attachment Protein (FAP) of MAP will facilitate Toll like Receptor 4 (TLR 4) to induce the dendritic cell to mature and activate T helper (Th1) cells (Lee *et al*., 2014). In 2013, a tuberculosis based vaccine delivery system was proposed based on nano-sized hepatitis B virus core protein particles (NHBc-VLP) (Dhanasooraj *et al*., 2013). *Mycobacterium tuberculosis* antigen culture filtrate protein 10 (CFP-10) was attached onto NHbc-VLP which facilitated an immune responsein Th1 dependent manner. With this concept, a MAP based vaccine delivery system could be proposed in which NHBc-VLP would bear MAP antigens that would facilitate the DC-mediated immune responses. Secretory protein like MAP1305 can be used for such studies as this protein modulate dendritic cell-mediated T cell proliferation through toll-like receptor-4 (Lee *et al*., 2014).

CONCLUSION

Effective treatment of JD is a major concern as its economic impact on farmer is devastating and its zoonotic potential is gaining ground among the scientific community. With culling of diseased animals being the most practised method of controlling the disease, nanotechnology offers a new insight into its therapeutic aspect. From drug delivery systems to nanobiotics, nanotechnology has been exploited to its fullest with compelling ideas and convincing results as an alternative to conventional method of treatment. Therapeutic approaches of nanotechnology based treatment for MAP related disease, offers a new insight into a more reliable, accurate and convenient method to stem the infection caused by MAP. Over the years nanotechology has gained signifiacnt role in the developmet of vaccine. They not only improve the immunogenicity and stability of the vaccines but they also facilitate target delivery and act as adjuvants, proving to be much more benefical than conventioal methods of vaccine fomulation. Though still at the investigational stage, they show huge potential to replace currently used methods as the single most effective method for treatment and vaccine development.

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