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Application of polymeric nanoparticles in oral delivery of recombinant human erythropoietin: A review

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ABSTRACT

Recombinant Human Erythropoietin drugs are known as erythropoietin stimulating agents which stimulate the bone marrow to produce more red blood cells in the body. It is used an antianemic in the treatment of renal anemia and chemotherapy induced anemia. It also use in treatment of HIV, cerebral malaria and neurological disease like schizophrenia. The recombinant human erythropoietin dosage form currently available in the market is parenteral dosage form that is ready for injection liquid vial (syringe), which is usually administered 2-3 times weekly. To achieve a therapeutic effect of parenterally administered EPO, cumulative doses are required that significantly exceed levels of endogenous EPO. These high serum levels result in prolonged circulation times of EPO and unspecific binding to non-targeted tissue, which may lead to severe undesired side effects i.e. growth of tumor and also increased risk of death. By using the nanotechnology, side effects and toxicity related to high dose of erythropoietin should be reduces and prolong drug release. this will achieve by reducing administration frequency and lowering dosage of erythropoietin.

Keywords: Recombinant Human Erythropoietin, Nanoparticle, Prolong drug release, Anemia

INTRODUCTION

Recombinant human erythropoietin (rHu EPO) is a 40% glycosylated protein hormone containing 165 amino acids. It is produced by the peritubular capillary endothelial cells in the kidney with a small amount being produced in the liver. EPO can be produced biosynthetically using recombinant DNA technology. Recombinant DNA technology has used to develop the synthetic forms of erythropoietin such as epoetin alfa, darbepoetin alfa, epoetin beta, epoetin delta, epoetin omega, and among the others.

EPO is considered as the world's best-selling drug with annual sales in the range 10–15 billion USD per year and it is currently used to help about 5% of the world population with renal failure and anemia. [1]   The global EPO drugs market is thus driven by increasing number of patients suffering from anemic condition induced due to cancer, HIV and ESRD treatment. Favorable reimbursements and increasing commercialization of EPO biosimilars the global Erythropoietin drugs market would reach $11.9 billion by 2020, registering CAGR of 9.7% during 2014-2020.

Uses of Erythropoietin: [2-3]

1) The main function of erythropoietin (EPO) is the regulation of erythropoiesis which is a long-term adaptation to boost O2-carrying capacity by increasing the number of RBCs and thus the haemoglobin concentration.

2) These erythropoietin (EPO) drugs are used for treatment of chemotherapy-induced anemia; anemia is a common side-effect occurring due to chemotherapy of End Stage Renal Diseases (ESRD)

3) Erythropoietin (EPO) is used for treatment of cancer and HIV.

4) EPO has been shown to also help certain neurological disease like schizophrenia.

5) Research has also suggested that EPO improves the survival rate for children suffering from cerebral malaria, which is caused by malaria parasite's blocking blood vessels in brain.

6) The most common use in people with anemia associated with abnormal function of the kidney.

GASTROINTESTINAL HURDLE IN ERYTHROPOIETIN ORAL DRUG DELIVERY

The administration of erythropoietin by the oral route has been the goal of many researchers. However, the oral bioavailability of erythropoietin is very poor. Macromolecular proteins normally cannot cross the intestinal epithelium. They will instead be degraded in the gastrointestinal tract before absorption[4]. Three main obstacles of erythropoietin oral delivery need to be considered:

1. Enzymatic barrier: The gastrointestinal tract has a variety of enzymatic barriers for erythropoietin oral delivery. The erythropoietin can be degraded by intracellular enzymes (e.g., cathepsins), bacterial flora in the mucus layer and in the epithelial cells of the intestine, and proteolytic enzymes in the stomach and in the intestinal lumen (e.g., pepsin, trypsin, and chymotrypsin) at the brush border membrane (e.g., endopeptidases). These enzymes denature protein drugs. Enzyme inhibitors can slow the erythropoietin degradation rate and increase the erythropoietin that is available for absorption. Sodium cholate and aprotinin reportedly act as enzyme inhibitors and improve protein absorption in rats [5].

2. Physiological barrier: The epithelial cells of the gastrointestinal tract are tightly bound by tight junctions (i.e., the zonulae occludentes) in which the outer surface of the intestinal epithelium is coated by mucus and glycocalyx layers, and thus inhibits the passage of erythropoietin and its subsequent absorption[6]. Absorption may be enhanced by using absorption enhancers such as bile salts, trisodium citrates, EDTA, labrasol, and polymeric materials (e.g., chitosan), which help to open the tight junctions of the intestinal epithelium [5, 7, 8].

3. Physicochemical properties of erythropoietin: The pore radius of the intestinal mucosa ranges 7e15A° which is an important barrier for macromolecular erythropoietin translocation. Temperature, solvents, and additives may disrupt the primary amino acid sequence and tertiary structure of protein. The alteration in the functional moiety or native charge of erythropoietin has an impact on its intestinal transport. At physiological pH, the carboxylic and amino groups of erythropoietin are entirely ionized, which results in a zwitterionic configuration. It is likely to preclude erythropoietin absorption from transcellular diffusion, unless the charges are neutralized through ion pairs [9]. The large molecular size of erythropoietin nevertheless remains an obstacle to its absorption. Modification of erythropoietin chemical structure against possible enzymatic degradation is an approach to raise its bioavailability. A diacyl derivative of some protein drug has been shown to maintain biological activity and to increase intestinal absorption of some protein drug [10].

DELIVERY SYSTEM FOR ERYTHROPOIETIN ORAL DELIVERY

Nanoparticles: Nanotechnology is a rapidly expanding field, encompassing the development of man-made materials in the 5-200 nanometer size range. The nanotechnology revolution has begun and shows enormous promise in the field of drug delivery. Nanotechnology has the potential to improve patient quality of life through decreased administrations due to longer drug release profiles and decreased side-effects due to reduced concentrations of toxic medications and targeted delivery. The ultimate drug delivery system that provides a universal platform for diagnosis, imaging and therapeutic treatment is another lofty goal that may be achieved through the application of nanotechnology. Based on the literature review of nanotechnology in drug delivery it was decided to proceed with the intention of developing a nanoparticle drug delivery system for the encapsulation and controlled release of rHu-EPO. Polymeric nanoparticles are selected as the most suitable due to their biocompatibility and non-toxic nature.[11] The bioavailability of orally delivered drugs is influenced by the physico-chemical properties of the drugs (i.e. solubility, pKa, size, etc.). Absorption of drug molecules depends on the particles in gastrointestinal tract (GIT) which occurs through various sites and depending upon their size. Particles size with 1 µm diameter are absorbed via phagocytosis by intestinal macrophages while particles <10 µm in diameter are transported through Peyer's patches (lymphatic islands present on GIT). Nanoparticles (<200 nm) are absorbed through endocytosis by enterocytes.[12] The efflux transporters such as P-glycoprotein (Pgp) and enzymes, expressed on enterocytes surface, also render the low systemic bioavailability of drugs affecting the absorption and excretion of drugs.[13] Nanotechnology reveals the application of size scale complex systems in various fields due to their unique properties.[14,15] One of the extensively studied areas of nanotechnology is delivering systems for the active ingredient of the medicine. Effective nanomedicine must be stable, biodegradable, non-toxic, non-inflammatory, non-thrombogenic, nonimmunogenic and should escape by reticuloendothelial system.[16,17] It has been proved experimentally that, for therapeutic and imaging applications, nanoparticles may range from 2 to 1000 nm should be applicable to different molecules such as small drugs, proteins, vaccines or nucleic acids. [18, 19]

APPROACHES FOR NANO PARTICULATE DRUG DELIVERY SYSTEM FOR ERYTHROPOIETIN

Nanocarriers have immense potential for the effective oral delivery of erythropoietin. Designing nanocarriers to improve erythropoietin gastrointestinal absorption may be achieved via modifying the polymer or nanoparticle surface property and applying an enteric coating onto the nanoparticles. These can be combined with enzyme inhibitors or absorption enhancers, as previously mentioned [20].

1. Polymeric nanocarrier approach: The polymeric nanoparticle is an approach to improve erythropoietin absorption from the gastrointestinal tract. Synthetic or natural polymeric materials modulate erythropoietin release and consequent pharmacological activity. Erythropoietin-loaded nanoparticles, which are prepared by using biodegradable polymers such as Chitosan, poly (lactide-co-glycolide), poly anhydride, and poly alkyl cyanoacrylate are absorbed from the intestinal epithelial cells and transport erythropoietin through the intestinal mucosa[20].

Incorporating erythropoietin in polymeric nanoparticle having the certain benefit like

➢ The drug incorporated is protected from biochemical degradation.
➢ Targeted delivery through enhanced permeability and retention.
➢ Extending in vivo half-life.
➢ Providing prolonged drug release; augmenting drug efficacy.
➢ Reducing side effects.
➢ Reducing administration frequency and lowering drug dosage.
➢ Enhance therapeutic effectiveness without frequent administration.
➢ Avoid poor patient compliance
➢ Reduction of drug toxicity & side effects.

2. Enteric coating approach: The enteric coating technique has been applied to erythropoietin oral delivery in which the enteric coating polymers possess a pH dependent property [21]. Polyacrylic polymers (e.g., Eudragit L100-55 and Eudragit S100) and cellulosic polymers (e.g., Hydroxy propyl methyl cellulose phthalate) have been widely used for this purpose [20, 21]. The increase in erythropoietin bioavailability is achieved by filling the freeze-dried chitosan/ poly (g-glutamic acid) (CS/g-PGA) nanoparticles in enteric coated capsules [22]. The enteric-coated capsules protect the erythropoietin-loaded nanoparticles from acidic gastric fluid and rapidly liberate erythropoietin in the proximal segment of the small intestine. Thus, the absorption of erythropoietin into systemic circulation is improved and the relative bioavailability of erythropoietin is increased.

3. Enzyme inhibitor approach: Erythropoietin, which is a protein, is easily digested and inactivated by digestive enzymes in the stomach after oral administration. Different protease inhibitors are administered along with the nanoparticles to inhibit the activity of these enzymes [24]. Radwan and Aboul-Enein[23] report that the oral administration of insulin (Protein drug) -loaded poly(ethylcyanoacrylate) nanoparticles in the presence of protease inhibitors (e.g., glycerrizin, capric acid, deoxycholic acid, hydroxypropyl-bcyclodextrin, and aprotinin) efficiently reduces and maintains glucose level < 200 mg/dL (i.e., the normal glucose level after a meal). Another approach to inhibit protease activity is by use of cationic metal chelating agents such as diethyl enetriamine penta acetic acid (DTPA)[24]. The addition of the complexing agent DTPA in insulin nanoparticles demonstrates a substantial protective effect against intestinal proteases in which the DTPA binds to cofactors [e.g., calcium (Ca2þ) and zinc (Zn2þ)] of the enzyme system and cause structural alterations and the loss of enzymatic activity.

4. Permeation enhancers approach: The absorption of protein drug from the gastrointestinal tract is improved by the coadministration of permeation enhancers that widen the intercellular junction (e.g., paracellular pathway) and/or perturbate the membrane phospholipids (e.g., transcellular pathway) [25]. Permeation enhancers which include fatty acids, surfactants, Ca2þ-chelating agents, and zonula occludens toxindare incorporated in the formulations. Another aspect is to conjugate with L-valine amino acid or any legand which are reported to improve the transport of protein drug across the epithelium cell of intestine. [26]

METHODS OF PREPARATION FOR NANOPARTICLES [27, 28]

1. Emulsion Solvent Evaporation Method: This method is use most frequently used methods for the preparation of nanoparticles. Emulsification solvent evaporation method includes two steps. First step involve emulsification of the polymer solution into an aqueous phase. In the second step polymer solvent is evaporated, and polymer precipitation as nanospheres. Nanoparticles are collected by using ultracentrifugation process.
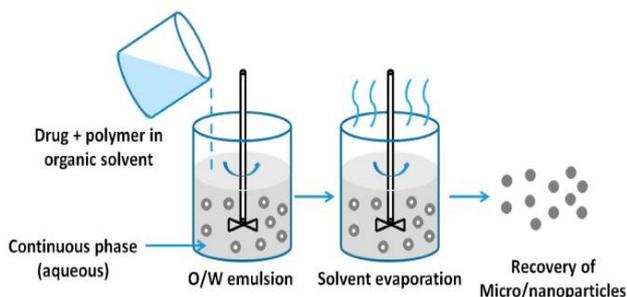

After centrifugation residue wash with distilled water. Size of nanoparticles can be control by adjusting the stirring speed, type and amount of dispersing agents, temperature, viscosity of organic and aqueous phases.

2. Double Emulsion and Evaporation Method: The emulsion and evaporation method which involves the addition of aqueous drug solutions into organic polymer solution under vigorous stirring to form w/o emulsions. Prepared w/o emulsion is then added into second aqueous phase with continuous stirring which form the w/o/w emulsion. This emulsion is subjected to solvent removal process by evaporation. Nanoparticles can be isolated by centrifugation at high speed. The prepared nanoparticles must be thoroughly washed and lyophilize.
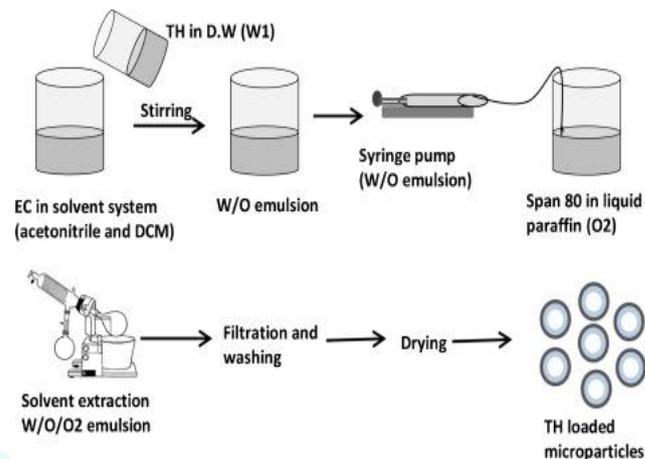

3. Emulsions Diffusion Method: In this method encapsulating polymer is dissolved in a partially water miscible solvent and then these solvent is saturated with water. The polymer-water saturated solvent phase is emulsified in an aqueous solution which contain stabilizer, leading to solvent diffusion to the external phase and the formation of nanocapsules or nanospheres.
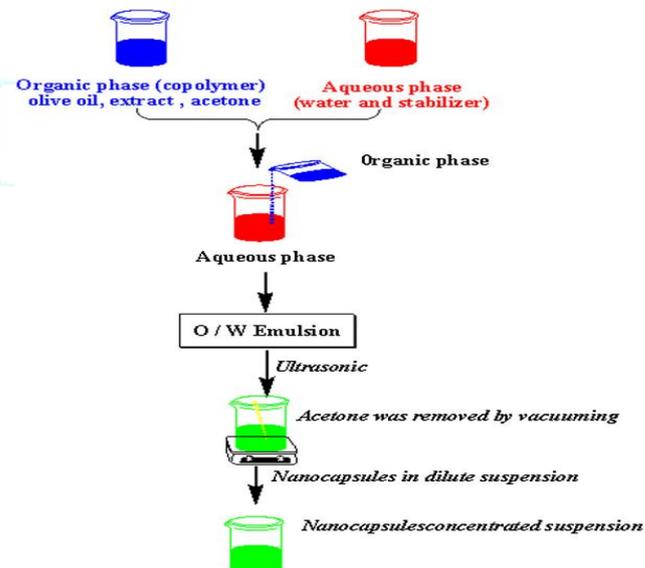

4. Solvent Displacement method: In this method precipitation of a polymer from organic solution and the diffusion of the organic solvent in the aqueous medium in the presence or absence of surfactant. Drug, Polymers, and/or lipophilic surfactant are dissolved in a water miscible semipolar solvent such as ethanol, acetone. The solution is then injected or poured into an aqueous solution under magnetic stirring. Nanoparticles prepared instantaneously by the rapid solvent diffusion. Then the solvent is removing from the suspension under reduced pressure.
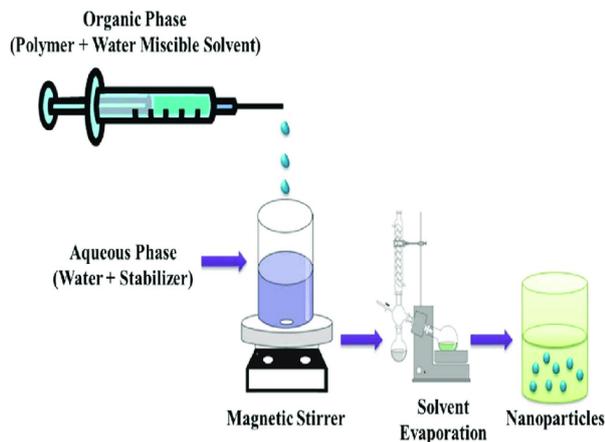
5. Reverse Micelles: Reverse micelles are mixtures of water, oil and surfactant which are thermodynamically stable and shows a dynamic behaviour. The structure of reverse micelles consists of aqueous and oil volumes which are separated by surfactant films, as the observer zooms out to a more macroscopic scale reverse micelles appear homogeneous and isotropic. This advantage of reverse micellar formation is that ultrafine polymeric nanoparticles with narrow size distributions are produced, while applying traditional emulsion polymerization method larger nanoparticles (>200nm) will form with broad size distribution. The reverse micelle aqueous core acts as a Nano reactor in preparation of ultrafine nanoparticles.
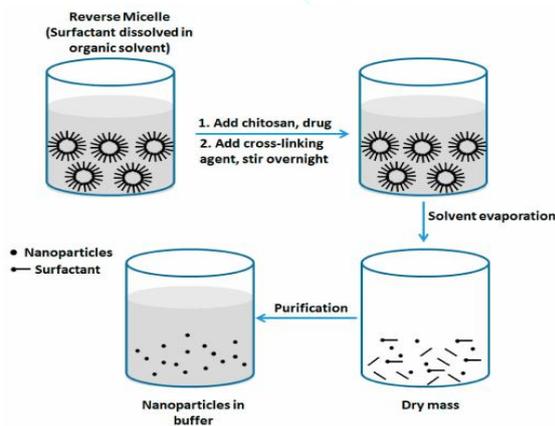
6. Ionotropic Gelation: In this process in which polyelectrolyte is cross-linked with a counter ion which forms a hydrogel. The structures of hydrogels are maintained by hydrogen bonding, hydrophobic forces, ionic forces or molecular entanglements. This technique has been applied using a variety of materials, including gellan gums, alginates, carboxymethyl cellulose and chitosan, to create micro and nanoparticles for encapsulation and controlled release of therapeutic agents.
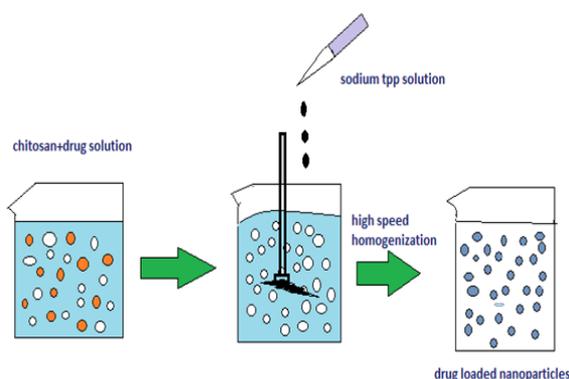
Depending on the material used, the strength of the counter ion and the desired particle size several methods of ionotropic gelation can be applied, including syringe dropping and air atomization for bead formation, and flush mixing for nanoparticles formation.

ABSORPTION MECHANISMS OF NANOPARTICLE DELIVERY SYSTEMS

The intestinal epithelium controls the passage of drugs, macromolecules, and particles. The surface area of the small intestine is enlarged because of the villi and microvilli which have a vital role in drug absorption in the gastrointestinal tract. The mechanisms of translocation of nanoparticles and micelles in the intestinal epithelium involve paracellular and/ or transcellular transport [29, 30] Paracellular pathway. The paracellular pathway is the preferred route for the transport of hydrophilic drugs. However, it restricts the passage of macromolecules or particles larger than approximately 1 nm because of a very small intercellular space and because of the tight junctions between the epithelial cells (pore diameter, 3e10 A°)[30]. Therefore, polymeric nanoparticles usually cannot pass through the intestinal barrier via the paracellular route. To improve their paracellular transport, the tight junction must be opened reversibly by using permeation enhancers such as cationic polymers (e.g., chitosan and its derivatives), anionic polymers (e.g., Polyacrylic acid and its derivatives), or calcium chelators (e.g., ethylene diamine tetra acetic acid). The width of the tight junction opened by the enhancers is < 20 nm, which still limits the transport of intact nanoparticles into the bloodstream if the nanoparticle is > 20 nm [31]. In this situation, the nanoparticles must be destabilized and disintegrated in the intercellular space when approaching the tight junction between the epithelial cells so that the loaded drug is released and permeated through the opened paracellular pathway.

BIOAVAILABILITY OF ERYTHROPOIETIN-LOADED NANOPARTICLES

The effectiveness and safety of erythropoietin nanoparticles have been assessed for their bioavailability, physiological response, therapeutic effect, and cytotoxicity. Venkatesan et al. prepared liquid-filled nanoparticles by using solid adsorbents such as carbon nanohorns and carbon nanotubes[32]. They found that liquid-filled erythropoietin carbon nanotubes improved the bioavailability of erythropoietin to 11.5% following invasive intra-small intestinal administration to rats. Bahgat E. Fayed et.al was loaded EPO in poly lactic-co glycolic acid (PLGA) nanoparticles which successfully altered the in vivo release profile and activity, allowing for more than 2-week activity after single injection using only double the EPO dose.[33] Thus due to entrapment of polypeptide drug in nanoparticles, the half-life of the drug may be increased and reduces the side-effects compared to conventional parenteral preparation. Thus the bioavailability of protein drug may improve with nanoparticulate technology.

CONCLUSION AND FUTURE PERSPECTIVE

It is very clear that the loading of EPO in nanoparticles successfully altered the in vivo release profile and activity, allowing for more than 2 week activity after single dose. Bahgat E. Fayed et.al was found that EPO in poly lactic-co glycolic acid (PLGA) nanoparticles improve release profile and activity, allowing for more than 2-week activity after single injection and reduces side effect due to drug accumulation[32]. Study found that in the treatment of anemia, hypoxia and in a newborn rat model, the effect of erythropoietin nanoparticles is 10 times greater than the

regular parenteral erythropoietin treatment[34] Cody Bulmer prepared the erythropoietin nanoparticles and showed 30% release of encapsulated erythropoietin in 24 h and 68% release in two weeks. These erythropoietin release results can be used as a basis for more research on the application of nanoparticles within animal models [35]. These studies show that erythropoietin loaded in nanoparticles significantly enhance its therapeutic effects. Further studies into the effect of nanoparticle structure on the biological activity of rHu-EPO and *in vivo* release studies need to be performed in order determine the true potential of nanoparticulate delivery for rHu-EPO for controlled release. The loading of EPO in nanoparticles depends upon the pH, Stirring speed, nature of polymer and a procedure use for the preparation of nanoparticles. Erythropoietin is protein structure which requires minimum stress conditions during preparation of nanoparticles to avoid any stability alteration to structure of erythropoietin.

Conflicts of interest: The authors have no conflicts of interest to declare.

REFERENCES

1. Core A, Hempel C, Kurtzhals JA, Penkowa M. Plasmodium berghei: erythropoietin activates neural stem cells in an experimental cerebral malaria model. Exp Parasitol, 2011; 127:2:500-505.

2. Tatkare D , Global Opportunity Analysis and Industry Forecast, 2015; 115:2013-2020

3. Mateja C, Barbara P, Porekar V, Gaberc S, Mateja N, Spela J, Radovan K, Simon C, Erythropoietin conjugates having oral bioavailability. https//www.google.com/patent/ 2015; 032981? cl= en & utm_source=gb-gplus-sharePatent WO2015032981A1

4. Woitiski CB, Carvalho RA, Ribeiro AJ, Neufeld RJ, Veiga F. Strategies toward the improved oral delivery of insulin nanoparticles via gastrointestinal uptake and translocation. BioDrugs 2008; 22:223e37.

5. Herrero EP, Alonso MJ, Saba N. Polymer-based oral peptide nanomedicines. Ther Deliv 2012; 3:657e68.

6. Damge C, Maincent P, Ubrich N. Oral delivery of insulin associated to polymeric nanoparticles in diabetic rats. J Control Release 2007; 117:163e70.

7. Li CL, Deng YJ. Oil-based formulations for oral delivery of insulin. J Pharm Pharmacol 2004; 56:1101e7.

8. Eaimtrakarn S, Rama Prasad YV, Ohno T, Konishi T, Yoshikawa Y, Shibata N, Takada K. Absorption enhancing effect of Labrasol on the intestinal absorption of insulin in rats. J Drug Target 2002; 10:255e60.

9. Damge C, Reis CP, Maincent P. Nanoparticle strategies for the oral delivery of insulin. Expert Opin Drug Deliv 2008; 5:45e68.

10. Kinesh VP, Neelam DP, Punit BP, Bhavesh SB, Pragna KS. Novel approaches for oral delivery of insulin and current status of oral insulin products. Int J Pharm Sci Nanotech 2010; 3:1058e64.

11. Patel J, Chauhan S, Seth A, Formulation & Evaluation of Methotrexate Loaded Nanoparticle. International Journal of Drug Discovery and Medical Research, 2012; 2:1:56-60.

12. Sharma G, Sharma A, Nam J, George P, Doss C, Lee S, Nanoparticle based insulin delivery system. Next generation efficient therapy for Type 1 diabetes. Journal of Nanobiotechnology 2015; 13:74.

13. Manthena V, Varma S, Ashokraj Y, Dey C, Panchagnula R, P-glycoprotein inhibitors and their screening: a perspective from bioavailability enhancement. Pharmacological Research, 2003, 48:4:347-359.

14. Singhal G, Bhavesh R., Kasariya K, et al., Biosynthesis of silver nanoparticles using Ocimum sanctum (Tulsi) leaf extract and screening its antimicrobial activity. Journal of Nanoparticle Research, 2011; 13:7:2981–2988.

15. Singhal G, Bhavesh R, Sharma A, Singh R, Ecofriendly Biosynthesis of Gold Nanoparticles Using Medicianally Important Ocimum basilicum Leaf Extract. Adv. Sci. Eng. Med, 2012; 4:62-66.

16. Kumari A, Yadav S, Yadav S, Biodegradable polymeric nanoparticles based drug delivery systems. Colloids and Surfaces B: Biointerfaces, 2010; 75:1–18.

17. Chakraborty C, Pal S, Doss GP, Wen ZH, Lin CS. Nanoparticles as smart pharmaceutical delivery. Front Biosci (Landmark Ed), 2013; 1:18:1030–1050.

18. Rieux A, Fievez V, Garinot M, Schneider YJ, Preat V. Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach. J Control Release, 2006; 116:1:1–27.

19. Singh R, Lillard JW, Nanoparticle based targeted drug delivery. Exp Mol Pathol, 2009; 86:3:215–223.

20. Woitiski CB, Carvalho RA, Ribeiro AJ, Neufeld RJ, Veiga F. Strategies toward the improved oral delivery of insulin nanoparticles via gastrointestinal uptake and translocation. BioDrugs 2008; 22:223e37.

21. Chen MC, Mi FL, Liao ZX, Hsiao CW, Sonaje K, Chung MF, Hsu LW, Sung HW. Recent advances in chitosan-based nanoparticles for oral delivery of macromolecules. Adv Drug Deliver Rev 2013; 6:865e79.

22. Sonaje K, Chen YJ, Chen HL, Wey SP, Juang JH, Nguyen HN, Hsu CW, Lin KJ, SungHW. Enteric-coated capsules filled with freeze-dried chitosan/poly(gamma-glutamic acid) nanoparticles for oral insulin delivery. Biomaterials 2010; 31:3384e94.

23. Radwan MA, Aboul-Enein HY. The effect of absorption enhancers on the initial degradation kinetics of insulin by alpha-chymotrypsin. Int J Pharm 2001; 217:111e20.

24. Su FY, Lin KJ, Sonaje K, Wey SP, Yen TC, Ho YC, Panda N, Chuang EY, Maiti B, Sung HW. Protease inhibition and absorption enhancement by functional nanoparticles for effective oral insulin delivery. Biomaterials 2012; 33:2801e11.

25. Park K, Kwon IC, Park K. Oral protein delivery: current status and future prospect. React Funct Polym 2011; 71:280e7.

26. Jain, A., & Jain, S. K. l-Valine appended PLGA nanoparticles for oral insulin delivery. Acta Diabetologica, 2015; 52(4):663–676

27. Fayed B, Tawfik AK, Yassin AE, Novel erythropoietin-loaded nanoparticles with prolonged in vivo response, Journal of Microencapsulation, 2012, 1–7.

28. Ehrenreich H, Degner D, Meller J, et al. Erythropoietin: a candidate compound for neuroprotection in schizophrenia. Molecular psychiatry, 2004; **9**(1):42–54.

29. Florence AT. Issues in oral nanoparticle drug carrier uptake and targeting. J Drug Target 2004; 12:65e70.

30. Chen MC, Sonaje K, Chen KJ, Sung HW, A review of the prospects for polymeric nanoparticle platforms in oral insulin delivery. Biomaterials 2011; 32:9826e38.

31. Sonaje K, Lin YH, Juang JH, Wey SP, Chen CT, Sung HW. In vivo evaluation of safety and efficacy of self-assembled nanoparticles for oral insulin delivery. Biomaterials 2009; 30:2329e39.

32. Venkatesan N, Yoshimitsu J, Lto Y, Shibata N. Liquid filled nanoparticles as a drug delivery tool for protein therapeutics. Biomaterials, 2005; 26:7154–63.

33. Fayed B. E., Tawfik A. F., & Yassin A. E. B. Novel erythropoietin-loaded nanoparticles with prolong release and vivo response. Journal of Microencapsulation, 2012; 29(7):650–656.

34. Chen H, Spagnoli F, Burris M, et al. Nanoerythropoietin is 10-times more effective than regular erythropoietin in neuroprotection in a neonatal rat model of hypoxia and ischemia. Stroke. 2012; 43:884–887.

35. Bulmer C., Margaritis A., & Xenocostas A. Production and characterization of novel chitosan nanoparticles for controlled release of rHu-Erythropoietin. Biochemical Engineering Journal, 2012; 68:61–69.