PLGA microspheres for sustained-release delivery of erythropoietin of native form to rhesus monkey, efficacy, pharmacokinetics, and immunogenicity

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ABSTRACT SUMMARY
We reported a method to formulate PLGA microspheres for sustained-release delivery of erythropoietin (EPO) in native form. The native conformation of EPO, which is easily altered and therefore induce severe antibody response, was preserved in glassy polysaccharide particles, ~1 μm in diameter, prior to microencapsulation. The polysaccharide particles, to which EPO was partitioned preferentially, were formed via an aqueous-aqueous emulsion or freezing induced phase separation to prevent proteins from contacting hydrophilic/hydrophobic interfaces, factors known to denature proteins. Pharmacokinetic and efficacy studies using rhesus monkeys showed sustained EPO concentration in blood and anti-anemic effect over two weeks after single dose of the microsphere formulation. Antibody assay of the blood samples from the monkeys received different formulations confirmed that the animals injected with our microspheres showed the same antibody level as those injected with saline and EPO solution. On the contrary, the monkeys injected with the microspheres formulated using the conventional double emulsion method had an antibody level more than ten times higher.

INTRODUCTION
Although long-dosing protein drugs based on biological or chemical structure modifications are available in the market, sustained-release dosage forms of native proteins are still meaningful, if succeed, for their longer and easily adjustable dosing period, technological versatility for applying to different proteins, better cost-efficiency as compared with the low yield PEGylation, and less R&D risk than biological altering because a protein analogue is often regarded as a new molecule. The challenges that retarded commercial success of sustained-release dosage forms of native proteins comprise (1) protein denaturing during the formulation process; (2) proteins denaturing during prolonged in vivo release; (3) initial burst release that compromises sustained efficacy; (4) incomplete release; and (5) unsatisfied encapsulation efficiency.

To address the issues above simultaneously, a formulation strategy involving pre-protection of proteins and stirring free microencapsulation was examined in the present study. EPO was selected as the model protein to examine the proposed process because of its known susceptibility to conformation hazards and antibody evoking. The steps and rationales of the formulation strategy are discussed below. First, delicate proteins (such as EPO) are loaded into fine dextran glassy particles via an aqueous-aqueous emulsion; this avoids exposing proteins to water-oil and water-air interfaces (factors known to denature proteins). Once loaded into the dextran particles, EPO becomes highly resistant to organic solvents and can therefore be microencapsulated in PLGA microspheres in a preserved native form. The EPO/dextran particles were suspended in a PLGA solution and squeezed through a porous glass membrane of designed pore size to form embryonic microspheres uniform in diameter. The formed microspheres were received by continuous phase which allowed the particles to pass through and solidify without stirring. This process reduced the chance of embryonic microspheres to fuse and break, which leads to over and under sized particles. After administration in vivo, the dispersed dextran particles absorb water and form a viscous phase around EPO; in this manner, protein aggregation and adsorption on the inner surface of PLGA microspheres (causes for incomplete release) can be avoided. In addition to protein protection, the viscous dextran phases also serve as selective diffusion channels in the PLGA matrix; this allows small buffer molecules to readily diffuse in and neutralize the acidity generated by PLGA degradation but retard burst release of the macromolecular proteins. Erythropoietin encapsulated into PLGA microspheres though the method was named as AqueEPO and assessed in rhesus monkey with anemia.

EXPERIMENTAL METHODS
AqueEPO were suspended in citric acid buffer and shaken at 37 ºC. The supernatant was collected at each sampling date and measured for protein concentration using an enzyme-linked immunosorbent assay (ELISA). The results were shown in Figure 1. The PK profiles of AqueEPO were also evaluated in monkeys by single injection of 30 μg/kg or 50 μg/kg; monkeys received single injection of empty microspheres served as control. The PK profiles obtained in monkeys were shown in Figure 2. Evaluation for in vivo efficacy of sustained-release AqueEPO was done in anemic monkey models. RBC, HGB, HCT and Retic were used as markers for EPO efficacy in this experiment. Reticulocytes and RBC of monkeys given 2 injections of SR microspheres at either high dose (2 x 15.3 = 30.6 μg/kg) or low dose (2 x 5.1 = 10.2 μg/kg) were compared to those given 12 injections of EPO solution (using 2-day injection intervals). Neutralizing anti-EPO antibody was detected in plasma of cynomolgus monkey at 6th week after subcutaneous injection though detecting residual EPO concentration in plasma via mixing with 100 IU/ml EPO standard solution with equal volume.
RESULTS AND DISCUSSION

The results shown in Figure 1 indicate that EPO was gradually released from the SR microspheres over 14 days and the first-day burst was limited to 20% of the total protein loading, which is comparable to the measures of SR peptide drugs and polyethylene glycol conjugated (PEGylated) proteins currently used in clinical practice.

![Figure 1](image1.png)

Figure 1. In-vitro release profiles of EPO from optimized SR PLGA microspheres. Mean ± SD. n=3.

The blood concentration of EPO reached to maximum level at 24 hours after administration and then steadily decreased, until it reached constant levels at Day 7 and kept stable for additional 7 or more days. The PK profiles obtained in monkeys were shown in Figure 2. The PK study results showed that a single injection of our microsphere formulation is able to maintain a sustained blood concentration of EPO for more than two weeks in rats and monkeys.

![Figure 2](image2.png)

Figure 2. Average plasma EPO concentration after subcutaneous injection of EPO loaded PLGA microspheres in cynomolgus monkey: 30μg rhEPO/kg (◆), 50 μg rhEPO/kg (▲) and the equivalent amount of blank PLGA microspheres without rhEPO (●). Mean ± SD. n=4.

The AqueEPO showed comparable efficacy on reticulocyte and RBC count, compared to EPO solution at 12 x 2.4 = 28.8 μg/kg, as shown in Figure 3. Hemoglobin (HGB) and hematocrit (HCT) were also measured in the samples and the results showed similar response curve by AqueEPO and EPO solution. Therefore, we concluded that by significantly less frequent injection, the in vivo efficacy of AqueEPO was comparable to that of EPO solution.

![Figure 3](image3.png)

Figure 3. Efficacy of sustained-release EPO microspheres in rhesus monkeys anemia. A: RBC(10^{12}/L); B: HGB(g/L); C: HCT; D: Retic(10^{9}/L).

Like other therapeutic proteins, AqueEPO is a biologics product and has the potential to be immunogenic in humans. In order to access the potential of AqueEPO to stimulate an immune response, anti-EPO antibody was determined by using the plasma sample from immunosuppressed cynomolgus monkey to neutralize fresh EPO.

![Figure 4](image4.png)

Figure 4. Comparison of anti-EPO antibody response after injection of our proposed SR EPO microspheres and the traditional microspheres.

CONCLUSION

As confirmed by efficacy, PK, and immunogenicity studies of sustained-release EPO using rhesus monkeys, the microsphere formulation process proposed in the present study (comprising pre-protecting protein and stirring shear-free encapsulation) may achieve safe and prolonged in vivo efficacy of protein drugs.

REFERENCE


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