basis of a pharmaceutical. For example, aniline has been shown to induce methemoglobinemia (Harrison, J.H.., and Jollow, D.J., Molecular Pharmacology, 32(3) 423-431, 1987). Long-term dietary treatment of rats has been shown to induce tumors in the spleen (Goodman, DG., et al., J Natl Cancer Inst., 73(1):265-73, 1984). In vitro studies have also shown that aniline has the potential to induce chromosome mutations and has the potentially genotoxic activity (Bombhard E.M. et Herbold B, Critical Reviews in Toxicology 35,783-835, 2005).

[0008] Considering the potentially dangerous properties of aniline and notwithstanding the methods available of conjugating water soluble polymers to therapeutic proteins, there remains a need to develop materials and methods for conjugating water soluble polymers to proteins that improves the protein's pharmacodynamic and/or pharmacokinetic properties while minimizing the costs associated with the various reagents and minimizing the health risks to the patient recipient.

SUMMARY ~~OF THE INVENTION~~

[0009] The present ~~invention~~ disclosure provides materials and methods for conjugating polymers to proteins that improves the protein's pharmacodynamic and/or pharmacokinetic properties while minimizing the costs associated with the various reagents and the health risks to the patient recipients when the conjugation reaction is catalyzed by a nucleophilic catalyst. In various embodiments of the present disclosure~~invention~~, alternative catalysts to substitute for aniline are provided.

[0009a] In a first aspect of the present disclosure, there is provided a method of conjugating a water soluble polymer to an oxidized carbohydrate moiety of a therapeutic protein comprising contacting the oxidized carbohydrate moiety with an activated water soluble polymer under conditions that allow conjugation;

said water soluble polymer containing an active aminooxy group and is selected from the group consisting of polyethylene glycol (PEG), branched PEG, PolyPEG® (Warwick Effect Polymers; Coventry, UK), polysialic acid (PSA), carbohydrate, polysaccharides, pullulane, chitosan, hyaluronic acid, chondroitin sulfate, dermatan sulfate, dextran, carboxymethyl-dextran, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polypropylene glycol (PPG), polyoxazoline, polyacryloylmorpholine, polyvinyl alcohol (PVA), polycarboxylate, polyvinylpyrrolidone, polyphosphazene, polyoxazoline,

polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, poly(1-hydroxymethylethylene hydroxymethylformal) (PHF), 2-methacryloyloxy-2'-ethyltrimethylammoniumphosphate (MPC); and

said carbohydrate moiety oxidized by incubation with a buffer comprising an oxidizing agent selected from the group consisting of sodium periodate ($NaIO_4$), lead tetraacetate ($Pb(OAc)_4$) and potassium perruthenate (KRuO4);

wherein an oxime linkage is formed between the oxidized carbohydrate moiety and the active aminooxy group on the water soluble polymer;

and wherein said oxime linkage formation is catalyzed by a nucleophilic catalyst m-toluidine.

[0009b] In a second aspect of the present disclosure, there is provided a method of forming an oxime linkage between an oxidized carbohydrate moiety on a therapeutic protein and an activated water soluble polymer containing an active aminooxy group comprising the steps of:

a) oxidizing a carbohydrate moiety on a therapeutic protein by incubating said protein with an oxidizing agent selected from the group consisting of sodium periodate ($NaIO_4$), lead tetraacetate ($Pb(OAc)_4$) and potassium perruthenate (KRuO4); and

b) forming an oxime linkage between the oxidized carbohydrate moiety of the therapeutic protein and the activated water soluble polymer containing an active aminooxy group in the presence of a nuclephilic catalyst under conditions allowing formation of said oxime linkage;

wherein said water soluble polymer containing an active aminooxy group is selected from the group consisting polyethylene glycol (PEG), branched PEG, PolyPEG® (Warwick Effect Polymers; Coventry, UK), polysialic acid (PSA), carbohydrate, polysaccharides, pullulane, chitosan, hyaluronic acid, chondroitin sulfate, dermatan sulfate, dextran, carboxymethyl-dextran, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polypropylene glycol (PPG), polyoxazoline, polyacryloylmorpholine, polyvinyl alcohol (PVA), polycarboxylate, polyvinylpyrrolidone, polyphosphazene, polyoxazoline, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, poly(1-hydroxymethylethylene hydroxymethylformal) (PHF), 2-methacryloyloxy-2'-ethyltrimethylammoniumphosphate (MPC);

wherein the nucleophilic catalyst is m-toluidine.

[0009c] In a third aspect of the present disclosure, there is provided a method of forming a hydrazone linkage between an oxidized carbohydrate moiety on a therapeutic protein and an activated water soluble polymer containing an active hydrazide group comprising the steps of:

a) oxidizing a carbohydrate moiety on a therapeutic protein by incubating said protein with an oxidinzing agent selected from the group consisting of sodium periodate ($NaIO_4$), lead tetraacetate ($Pb(OAc)_4$) and potassium perruthenate (KRuO4); and

b) forming a hydrazone linkage between the oxidized carbohydrate moiety of the therapeutic protein and the activated water soluble polymer containing an an active hydrazide group in the presence of a nuclephilic catalyst under conditions allowing formation of said hydrazone linkage;

wherein said water soluble polymer containing an active hydrazide group is selected from the group consisting of polyethylene glycol (PEG), branched PEG, PolyPEG® (Warwick Effect Polymers; Coventry, UK), polysialic acid (PSA), carbohydrate, polysaccharides, pullulane,chitosan, hyaluronic acid, chondroitin sulfate, dermatan sulfate, dextran, carboxymethyl-dextran, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polypropylene glycol (PPG), polyoxazoline, polyacryloylmorpholine, polyvinyl alcohol (PVA), polycarboxylate, polyvinylpyrrolidone, polyphosphazene, polyoxazoline, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, poly(1-hydroxymethylethylene hydroxymethylformal) (PHF), 2-methacryloyloxy-2'-ethyltrimethylammoniumphosphate (MPC);

wherein the nucleophilic catalyst is m-toluidine.

[0010] In one embodiment, a method of conjugating a water soluble polymer to an oxidized carbohydrate moiety of a therapeutic protein is provided comprising contacting the oxidized carbohydrate moiety with an activated water soluble polymer under conditions that allow conjugation; said water soluble polymer containing an active aminooxy group and is selected from the group consisting of polyethylene glycol (PEG), branched PEG, PolyPEG® (Warwick Effect Polymers; Coventry, UK), polysialic acid (PSA), starch, hydroxyalkyl starch (HAS), hydroxylethyl starch (HES), carbohydrate, polysaccharides, pullulane, chitosan, hyaluronic acid, chondroitin sulfate, dermatan sulfate, starch, dextran, carboxymethyl-dextran, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polypropylene glycol (PPG), polyoxazoline, polyacryloylmorpholine, polyvinyl alcohol (PVA), polycarboxylate, polyvinylpyrrolidone, polyphosphazene, polyoxazoline, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, poly(1-

15. The method according to any one of claims 1-14 wherein the oxidizing agent is $NaIO_4$.

16. The method according to any one of claims 1-15 wherein the nucleophilic catalyst is provided at a concentration between about 1 mM and about 50 mM; preferably at a concentration of about 10 mM.

17. The method according to any one of claims 1-16 further comprising the step of reducing an oxime linkage in the conjugated therapeutic protein by incubating the conjugated therapeutic protein in a buffer comprising a reducing compound selected from the group consisting of sodium cyanoborohydride ($NaCNBH_3$), ascorbic acid (vitamin C) and $NaBH_3$.

18. The method according to any one of claims 1-17 further comprising the step of purifying the conjugated therapeutic protein; for example;

wherein the chromatography is hydrophobic interaction chromatography (HIC); wherein the one or more washing steps flow rate is about 2 cm/min; and wherein the one or more elution steps flow rate is about 1 cm/min.

25. A method of forming an oxime linkage between an oxidized carbohydrate moiety on a therapeutic protein and an activated water soluble polymer containing an active aminooxy group comprising the steps of:

a) oxidizing a carbohydrate moiety on a therapeutic protein by incubating said protein with an oxidizing agent selected from the group consisting of sodium periodate ($NaIO_4$), lead tetraacetate ($Pb(OAc)_4$) and potassium perruthenate (KRuO4); and

b) forming an oxime linkage between the oxidized carbohydrate moiety of the therapeutic protein and the activated water soluble polymer containing an active aminooxy group in the presence of a nuclephilic catalyst under conditions allowing formation of said oxime linkage;

wherein said water soluble polymer containing an active aminooxy group is selected from the group consisting polyethylene glycol (PEG), branched PEG, PolyPEG® (Warwick Effect Polymers; Coventry, UK), polysialic acid (PSA), carbohydrate, polysaccharides, pullulane, chitosan, hyaluronic acid, chondroitin sulfate, dermatan sulfate, dextran, carboxymethyl-dextran, polyalkylene oxide (PAO), polyalkylene glycol (PAG), polypropylene glycol (PPG), polyoxazoline, polyacryloylmorpholine, polyvinyl alcohol (PVA), polycarboxylate, polyvinylpyrrolidone, polyphosphazene, polyoxazoline, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, poly(1-hydroxymethylethylene hydroxymethylformal) (PHF), 2-methacryloyloxy-2'-ethyltrimethylammoniumphosphate (MPC);

wherein the nucleophilic catalyst is m-toluidine.