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Mannosamine–biotin as a novel masking agent for coating IgG for immune response silencing and augmentation of antibody–antigen interaction

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ABSTRACT

A variety of protein-coating procedures are used to modify proteins' properties. The principle coating agent used is PEGylation, in which proteins are coated by conjunction to polyethylene glycol (PEG). In the present study, we describe a novel approach that makes use of small molecules with multifunctional groups as the protein-coating agent. The new coating molecule was produced by reacting two endogenous molecules, mannosamine and biotin, to form mannose–biotin adducts (MBA). hIgG was coated with MBA at various MBA/protein ratios. The immunogenicity of MBA-coated hIgG was tested in chickens. A dose-responsive effect of MBA/hIgG ratio on immune response suppression was detected, with an optimal masking effect at a 12:1 ratio. The immune response to MBA-coated hIgG was about eightfold lower than that to PEG-coated hIgG. MBA also increased antibody–antigen-binding affinity, and decreased recognition of the Fc domain of MBA-coated hIgG by Fc receptor and secondary antibodies. While the PEG molecule consists of inert repeating units of ethylene oxide with no additional functional group to allow for potentially desirable modifications, the MBA has several functional groups, including vicinal hydroxyls, which can easily be converted to active residues such as aldehydes or carboxyls. This may be of importance for developing passive immunizations or for achieving tolerance of the immune response to an immunogenic molecule or virus. In summary, we developed a new protein-coating molecule with the ability to mask foreign antigens and in the case of antibodies, to enhance activity.

1. Introduction

A variety of methodologies are in wide use for coating drugs and therapeutic proteins in order to alter their properties. Such modifications include: changing a drug's pharmacokinetic parameters of absorption, biodistribution, metabolism and elimination [1], changing its molecular size, generally via attachment of a high-molecular-weight (MW) coating agent (e.g. polymers), and changing its structure and surface properties by forming barriers to its surroundings which, can affect the drug's polarity, as well as its adhesion to cells and tissues. Such modifications are aimed at maintaining or improving the drug's activity [1], by influencing its characteristic biological behavior, stability, immunogenicity, cellular uptake, biotransformation, excretion or tissue-localization profile. The major coating agent currently in use for drugs is PEG. In the process of PEGylation, the polymer is covalently bound to the drug either irreversibly or via reversible links which can be hydrolyzed in vivo, releasing the PEG moiety from the drug. Such bond formation requires modification and activation of the hydroxyl end of the PEG molecule and many irreversible and reversible modifications have been [2,3], and continue to be [4] developed. PEGylation has been used to modify a variety of proteins with clinical applications; these modifications are aimed mostly at increasing the protein's life span in the circulation by decreasing its total body clearance, improving its penetration into a target site and increasing its bioavailability. Examples of PEGylated proteins include interferon-α-2b [5] for application in hepatitis C and tumors [6], interleukin-2 [7], the staphylokinase variant SY161-P5 [8], tumor necrosis factor alpha for mediating acute and chronic inflammation [9], and the enzyme superoxide dismutase for increased serum persistence and reduced immunogenicity [10]. These PEGylated proteins have improved pharmacological properties, and in many instances are associated with a significant reduction in protein activity [11]. Polysaccharides—mostly dex-

Abbreviations: MBA, mannosamine–biotin adduct; PEG, polyethylene glycol; hIgG, human IgG; TT, tetanus toxoid; MPEG-NHS, methoxy polyethyleneglycolsuccinate *N*-hydroxysuccinimide; TNBSA, trinitrobenzenesulfonic acid or picrylsulfonic acid; TNP, trinitrophenyl; ECL, enhanced chemiluminescence; FCAIFA, Freund's complete adjuvant, incomplete Freund's adjuvant; CFDE, carboxyfluorescein succinimidyl ester; HRP, horseradish peroxidase.

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trans or modified dextrans of various MWs, Ficoll and polyvinyl alcohol, have also been used for protein modification. Examples include the formation of β-lactoglobulin with reduced immunogenicity [12], and the use of dextran, pullulan and mannan (a mannose polymer) to improve therapeutic agent delivery [13].

An occasional drawback of protein PEGylation is a significant reduction in the binding affinity of the modified protein to its target (receptor) [8,11]. Furthermore, the use of PEGylated drugs in vivo releases a non-biodegradable PEG molecule which, when the drug it coats is prescribed routinely or in large doses, may limit its pharmaceutical utilization [14]. Furthermore, large-scale PEGylation of proteins requires substantial technical and economic resources in the downstream purification stage, where excess PEG and uncoated protein have to be removed from the coated molecules. From a chemical perspective, the PEG molecule is characterized by a linear structure made up of inert repeat units of ethylene oxide with no additional functional group to allow for additional, potentially desirable modifications.

Our principle hypothesis in the present study was that the immune response to a particular antigen can be controlled to some extent by reacting it chemically with specific, preferentially endogenous agents. The selection of such agents is a key step in controlling the desired biological effect. In this respect, it is essential that during the process of changing the properties of the designed molecule (protein), the active site remain unaffected, at least retaining its original efficiency. The current study was aimed at developing methods for decreasing the antigenicity of foreign antibodies by coating them with small, non-immunogenic molecules. Such modifications should not affect the antibody's binding site and must allow its use for passive immunization in repeat treatments without triggering the development of an immune response. In the present study, we report our in vitro and in vivo results on coating antibodies with low-MW molecules.

2. Materials and methods

2.1. Materials

Human (h) IgG was purified from the whole serum of patients immunized with tetanus toxoid (TT). D-Mannosamine, $NaCNBH_3$, MPEG-NHS (methoxy polyethyleneglycolsuccinate *N*-hydroxysuccinimide) and biotin were purchased from Sigma–Aldrich. Biotin-NHS was purchased from Pierce. Amicon ultra-centrifugal filter devices (MWCO 10,000 and 30,000) were purchased from Millipore.

2.2. Synthesis of mannose–biotin (MBA)

2-D-Mannosamine (70 mg, 0.39 mmol) was dissolved in 0.5 ml DMSO and biotin-NHS (100 mg, 0.3 mmol) was added. The solution was stirred at room temperature (RT) for 2 h. A new peak was formed as detected by HPLC at a retention time of 14 min (for details see HPLC analysis). The new peak was isolated and purified by flash chromatography (silica gel, methanol:ethyl acetate, 5:95 as solvents). Using conventional analytical methods, the pure product was identified as MBA. The LC–MS of the product, using positive ion monitoring mode (ES^+), revealed the expected molecular ion *m*/*z* of 406 ($M+H^+$) and fragmentations with *m*/*z* of 364.6 and 249.5, identical to previously reported data (Lin, Chun-Cheng et al., Tetrahedron lett.1997, 38, 2649).

2.3. HPLC analysis of MBA

The HPLC was connected to a diode array detector (HP-1100) and equipped with a reverse-phase column (C-18, 150 mm length; 4.6 mm diameter with 5 μm particles). The mobile phase was a mixture of acetonitrile and water which was run at a flow rate of 1 ml/min with the following gradient: acetonitrile from 1% to 5% over 5 min, and then to 20% for another 5 min and finally to 98% for an additional 10 min.

2.4. LC/MS/MS analysis of MBA

The product was injected into MS in a direct injection with scan, using the ESI^+ method. The source temperature of the MS was set at 150 °C, with a cone gas flow of 22 l/h, a desolvation gas flow of 400 l/h and a capillary voltage of 3.5 kV. Peak spectra were monitored between 30 and 800 *m*/*z*.

2.5. Coating of hIgG antibody with MBA

MBA (1 mg, 2.5 μmol in 60 μl DMSO) was added to hIgG (1 mg, 6.6 nmol in 1 ml of 25 mM phosphate buffer (PB), pH 6). The solution was mixed for 1 h at RT, and then $NaCNBH_3$ (2 mg, 32 μmol) was added and the reaction was continued for an additional 2 h at RT. Excess MBA reagent was discarded from the reaction solution by filtration through an Amicon ultra-centrifugal filter device with a MWCO of 10,000.

2.6. Controlling the coating reaction (MBA/hIgG ratio)

Various amounts of MBA (20, 80, 300 and 1000 μg) from a stock solution of 25 mg in 1 ml DMSO were added to hIgG (1 mg hIgG in 1 ml of 25 mM (PB), pH 6) to monitor the ratio of MBA/hIgG (coating/coated ratio). The solution was mixed for 1 h at RT and different amounts of $NaCNBH_3$ (30, 120, 450 and 1500 μg from a stock solution of 17 mg $NaCNBH_3$ in 1 ml PBS) were added, respectively. The solution was left for another 2 h at RT. The excess MBA was then removed from the reaction mixture by filtration through an Amicon ultra-centrifugal filter device (MWCO 10,000). This filtrate was then taken for further analysis.

2.7. Coating hIgG with methoxy-PEG-NHS

MPEG-NHS (7 mg) was added to hIgG (1 mg in 1 ml PBS pH 7.4). The solution was mixed for 3 h at RT and then excess reagent was removed by filtration through an Amicon ultra-centrifugal filter device (MWCO 30,000). The filtrate was then taken for further analysis.

2.8. Number of unbound free amino groups in the coated and uncoated protein

The following procedure was based on a previous work [15] with some modifications. Briefly, the coated and uncoated hIgG were reacted with TNBSA (trinitrobenzenesulfonic acid or picrylsulfonic acid). Under mild conditions, this reagent reacts specifically with free amino groups on the amino acid side chain of a protein to give trinitrophenyl (TNP) derivatives. Thus, 50 μg of hIgG (from a stock solution of 1 mg/ml in PBS) was added to 140 μl sodium tetraborate buffer (0.1 M, pH 9.3) in a 96-well plate. Aqueous TNBSA (10 μl of 0.01 M) was added and the solution was incubated for 30 min at 37 °C. The absorption of the solution was measured at 405 nm in an ELISA reader (Lumitron) and the amount of free amine was calculated from a calibration curve prepared by reacting TNBSA with a known amount of glycine.

2.9. Gel electrophoresis and western blot analysis

To determine changes in the size of hIgG after modification with various amounts of MBA, the samples were analyzed by SDS-PAGE.