Pharmaceutical nanotechnology

Production, purification and biological characterization of mono-PEGylated anti-IL-17A antibody fragments

Salome-Juliette Koussoroplis a, Sam Heywood b, Catherine Uyttenhove c,d, Céline Barilly a, Jacques Van Snick c,e, Rita Vanbever a,∗

a Pharmacetics and Drug Delivery Group, Louvain Drug Research Institute, Université catholique de Louvain, Brussels 1200, Belgium
b UCB Pharma, Slough Sl1 4EN, United Kingdom
c Ludwig Institute for Cancer Research, Brussels 1200, Belgium
d Cellular Genetics Unit, du Deve Institute, Université catholique de Louvain, Brussels 1200, Belgium
e Experimental Medicine Institute, de Duve Institute, Université catholique de Louvain, Brussels 1200, Belgium

A R T I C L E   I N F O
Article history:
Received 17 May 2013
Received in revised form 19 June 2013
Accepted 27 June 2013
Available online 9 July 2013

Keywords:
Antibody fragment
PEGylation
Anti-interleukin-17A
Bronchial hyperreactivity

A B S T R A C T
The aim of this study was to maximize the yield of the production of mono-PEGylated anti-interleukin-17A (anti-IL-17A) antibody fragments using large (≥20 kDa) polyethylene glycol (PEG) chains. Particular attention was paid to selectivity yield mono-PEGylated species to maintain the maximum possible functionality and to simplify the purification. Neutralization of IL-17A by antibody constructs might find application for the treatment of bronchial hyperreactivity. Amino-directed and sulfhydryl-directed PEGylation of the native antibody fragments were compared. The former was selected as it produced the most interesting construct in terms of yield and preservation of biological activity. In particular, the R(AB)2-PEG conjugate with one 40 kDa branched PEG prepared in this study was produced at a 42% yield. The conjugate presented only a slight decrease in its binding activity and in its in vitro inhibitory potency offering interesting perspectives for in vivo studies.

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1. Introduction

Monoclonal antibodies (mAbs) are of particular therapeutic interest due to their high specificity in binding to their target molecules. Numerous mAbs are currently used clinically or undergoing late-stage clinical trials (Chan and Carter, 2010).

Recombinant antibody fragments (Fab′) with a single hinge cysteine, scFv, single V-type domains antibodies, diabodies, triabodies, minibodies) can provide an effective alternative to full length antibodies (Holliger and Hudson, 2005). Among others, the advantages of antibody fragments are the lack of Fc-dependent inflammation, enhanced tumor and tissue penetration (King et al., 1994; Oh et al., 2004), penetration into inaccessible antigen sites (Stijlumans et al., 2004) as well as increased avidity and multispecific action (Casey et al., 2002; Griffiths et al., 2004; Weir et al., 2002). Furthermore, antibody fragments can be economically produced in bacterial fermentation in contrast to full length antibodies which require mammalian cultures with lower capacity (Chapman, 2002; Weisser and Hall, 2009). Yet, a main drawback of antibody fragments is the short residence time in intact form in the body. This is in part due to the fact that they have a molecular weight below the kidney filtration threshold (∼60 kDa) but also due to the lack of recycling by the neonatal Fc receptor (FcRn)-mediated recycling pathway (Roopenian and Akilesh, 2007).

PEGylation of proteins is a common approach to increase serum half-life (Knight et al., 2004; Kontermann, 2009; Koumenis et al., 2000; Leong et al., 2001). The conjugation of PEG chains to proteins and antibody fragments may prolong the residence in the body due to reduced renal clearance, enhanced proteolytic resistance and reduced recognition by specific antibodies (Bailon and Won, 2009; Pasut et al., 2004; Veronese and Pasut, 2005).

Proteins attached to PEG chains ranging from 20 to 40 kDa are believed to be sufficiently large to overcome kidney ultrafiltration and to obtain a clinically useful circulation half-life (Jorgensen and Moller, 1979). Single- and short-chain PEGs usually fail to yield sufficient steric protection of large proteins (Park et al., 2010). In the case of IgGs (∼150 kDa) or large IgG fragments, the molecular

Abbreviations: PEG, polyethylene glycol; mAbs, monoclonal antibodies; AUC, area under the curve; BHR, bronchial hyperreactivity; HSFM, hybridoma serum free medium; LPS, lipopolysaccharide; PBBS, phosphate buffered saline; PVDF, polyvinylidene fluoride; FPLC, fast protein liquid chromatography; GFC, gel filtration chromatography; CEC, cation exchange chromatography; 2-MEA, 2-mercaptoethanolamine HC1; GES, glycine buffered saline; FCS, fetal calf serum; NHS, N-hydroxysuccinimid; GCSF, granulocyte colony-stimulating factor.

∗ Corresponding author at: Université catholique de Louvain, Louvain Drug Research Institute, Pharmacetics and Drug Delivery Group, Avenue Emmanuel Mounier 73, Brussels 1200, Belgium. Tel.: +32 2 764 73 25; fax: +32 2 764 73 98.
E-mail address: rita.vanbever@uclouvain.be (R. Vanbever).

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http://dx.doi.org/10.1016/j.ijpharm.2013.06.077
weight is above the glomerular filtration limit (70 kDa for globular proteins), therefore, PEGylation might not offer retarded renal clearance (Bailon and Won, 2009). Large proteins with a molecular weight above 70 kDa are chiefly eliminated from the body via alternative routes such as liver uptake, degradation by proteolytic enzymes and clearance by the immune system (Caliceti and Veronese, 2003). Addition of a single branched 40 kDa PEG to a F(\(ab\)\(^{\prime}\)\(^{2}\)) anti-IL-8 antibody fragment led to approximately 5-fold increase in serum half-life and an 16-fold increase in area under the curve (AUC) after intravenous administration as compared to the unmodified protein. Furthermore, the attachment of two 40 kDa PEG molecules to the same antibody fragment led to an AUC which was about 18-fold higher than the unmodified protein (Koumenis et al., 2000). Even though the unconjugated constructs were exceeding the kidney ultrafiltration limit, PEGylation had an impact on the residence time in the bloodstream, indicating that it also affects the alternative routes of protein catabolism.

In this study, PEGylation methods for murine anti-IL-17A Fab\(^{\prime}\) and F(\(ab\)\(^{\prime}\)\(^{2}\)) antibody fragments are presented. Interleukins (IL)-17 contribute to bronchial hyperreactivity (BHR), mucus secretion and inflammation suggesting that they play a pivotal role in asthma pathogenesis (Barczyk et al., 2003; Nembrini et al., 2009). Therefore, the inhibition of IL-17 by anti-cytokine antibodies (anti-IL-17) may offer a new opportunity to target mechanisms of asthma. A single large PEG chain (\(\geq 20\) kDa) was attached to Fab\(^{\prime}\) or F(\(ab\)\(^{\prime}\)\(^{2}\)) antibody fragments to produce conjugates retaining the maximum possible functionality and which can be easily purified. The generated products were purified, characterized and their biological activities were assessed in vitro.

2. Materials and methods

2.1. Materials

Linear 5 and 20 kDa maleimide PEG (abbreviated as PEG5-mal and PEG20-mal, respectively) were obtained from Creative PEGworks (Winston Salem, NC, USA). Linear 20 kDa and branched 40 kDa N-hydroxysuccinimide-PEG (abbreviated as PEG20-NHS and PEG40-NHS, respectively) purchased from NOF Corporation (Tokyo, Japan). Unless otherwise stated, chemicals and reagents were from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Production and purification of anti-IL-17A

Anti-IL-17A hybridoma (MM17F3, IgG1-kappa) was derived from mice vaccinated with mouse IL-17A conjugated to ovalbumin (Uyttenhove and Van Snick, 2006). Hybridoma cells were cultured in hybridoma serum free medium (HSFM; Invitrogen, Carlsbad, CA, USA) supplemented with IL-6 (1 ng/ml). The antibody was purified by passage over a Protein G Sepharose\(^{\text{TM}}\) 4 Fast Flow column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and eluted with 0.1 M glycine–HCl buffer pH 2.8. Eluted antibody was collected in tubes containing 1 M Tris–HCl buffer pH 8 for immediate neutralization. Lipopolysaccharide (LPS) traces were removed by passage over Sartobind\(^{\text{®}}\) IEC MA 15 (Sartorius-stedium biotech GmbH, Goettingen, Germany). Purified antibody was concentrated and dialyzed against phosphate buffered saline (PBS) before use.

2.3. Fast protein liquid chromatography (FPLC)

Chromatography was performed on an ÄKTAM\(^{\text{TM}}\) purifier 10 system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Absorbance at 280 nm was monitored where PEG has minimal signal contribution. The samples were filtered through a polyvinylidene fluoride (PVDF) syringe-tip filter of 0.2 \(\mu\)m prior to loading. Data were recorded by UNICORN software (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Product yields were calculated by the comparison of the relative area under the peak of each molecular species.

A HiLoad 16/60 Superdex 200 pg column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used for purification of the F(\(ab\)\(^{\prime}\)\(^{2}\)) and the Fab\(^{\prime}\) antibody fragments by gel filtration chromatography (GFC). The mobile phase was PBS and the elution was isocratic. The flow rate was 0.8 ml/min.

The diverse PEGylated products were purified by cation exchange chromatography (CEC) on a Resource S, 1 ml column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). A salt gradient elution was used. Buffer A was 20 mM CH\(_3\)COONa, 5 mM NaCl, pH 4.7 and buffer B was 20 mM CH\(_3\)COONa, 350 mM NaCl, pH 4.6. The column had a maximum capacity of 25 mg. To reduce interference of excess PEG with the column medium about 0.25 mg of protein was loaded each time.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with PEG staining

SDS-PAGE was performed using a gradient 4–20% Mini-PROTEAN\(^{\text{TM}}\) TGX\(^{\text{TM}}\) precast gel (Bio-Rad, Hercules, CA, USA) along with custom made buffer containing 0.025 M tris(hydroxymethyl)aminomethan, 0.192 M glycine and 0.1% sodium dodecyl sulfate, pH 8.7. Ten \(\mu\)g of protein was applied in each well, and the gels were run at 125 V, constant voltage, for 110 min. The protein bands were visualized by staining with GelCode\(^{\®}\) Blue Stain Reagent (Thermo Fisher Scientific, Rockford, IL, USA). PEG species were specifically stained with a barium iodide solution based on Kurfürst’s method (Kurfürst, 1992). Briefly, the gel was soaked in 0.1 M HClO\(_4\) for 30 min and then immersed in a 5% BaCl\(_2\) in 1 M HCl solution for 10 min. Then, iodine solution (1.3 I\(_2\) + 4% KI) was added and incubated for 5 min. Finally, the gel was left to destain in 0.05 M HCl for 30 min.

2.5. Production of F(\(ab\)\(^{\prime}\)\(^{2}\))

Anti-IL-17A (4 mg/ml) at a w/w [enzyme]:[antibody] ratio of 1:20 in 20 mM CH\(_3\)COONa buffer, pH 4 was incubated with pepsin at 37°C for 39 h. The reaction was quenched by addition of 2 M Tris base, pH 7.5. The mixture was then analyzed by SDS-PAGE and the F(\(ab\)\(^{\prime}\)\(^{2}\)) fragment was purified by GFC. The eluted fractions were further analyzed by SDS-PAGE to confirm the size and the purity. The concentration of the final product was measured by absorbance at 280 nm on a NanoDrop spectrophotometer 2000 (Fisher Scientific, Waltham, MA, USA) using an extinction coefficient of 1.25. The procedure described was highly reproducible and generated F(\(ab\)\(^{\prime}\)\(^{2}\)) at a yield of 92%.

2.6. Preparation of PEG-Fab\(^{\prime}\) via sulphydryl group-directed PEGylation

Prior to the conjugation reaction, the anti-IL-17A F(\(ab\)\(^{\prime}\)\(^{2}\)) fragment (1 mg/ml) was treated with 10 mM 2-mercaptoethamine-HCl (2-MEA) in 100 mM sodium phosphate, 150 mM NaCl, 8 mM EDTA, pH 7.2 at 37°C for 90 min to cleave the inter-heavy disulfide bonds. The reaction mixture was cooled to room temperature. To remove the 2-MEA excess, the solution was loaded to a PD-10 desalting column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), pre-equilibrated with 100 mM sodium phosphate buffer, 2 mM EDTA, pH 6.8. Elution was then performed using the same buffer and 0.5 ml fractions were collected. All fractions were measured at 280 nm in order to identify which ones contained the Fab\(^{\prime}\) construct. An Ellman’s thiol assay (Thermo Fisher Scientific, Rockford, IL, USA) was then performed to all of the fractions according to the manufacturer’s instructions to
distinguish the aliquots containing the Fab' thiols from the one having the reductant 2-MEA thiols. The relative amounts of \( F(ab')_2 \) and Fab' were determined by FPLC using a HiLoad 16/60 Superdex 200 pg column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and 100 mM sodium phosphate buffer, 2 mM EDTA, pH 6.8 for the mobile phase at 0.8 ml/min. Fractions containing Fab' with free sulfhydryl groups were pooled and immediately prepared for reaction with PEG-maleimide. More precisely, a solution containing 1 mg/ml of Fab' in 100 mM sodium phosphate buffer, 2 mM EDTA, pH 6.8 was mixed with linear PEG-maleimide (PEG5-mal or PEG20-mal). For the PEG conjugation, different molar ratios of [PEG]:[Fab'] ranging from 0.6:1 to 75:1 were tested. In all cases, the reaction was performed at ambient temperature, under agitation, overnight. The mixture was analyzed by SDS-PAGE and the gels were stained with both GelCode Blue Stain Reagent and barium iodide stain to assess the extent of PEGylation.

2.7. Preparation of PEG-\( F(ab')_2 \) via amine group directed PEGylation

Factors affecting the degree and the yield of PEGylation, including pH, incubation time, [PEG]:[fragment] molar ratio and protein concentration were evaluated. Best conditions tested were observed when anti-IL-17A F(\( ab')_2 \) (4 mg/ml) in 50 mM HEPES, pH 8.2 was allowed to react with branched PEG40-NHS activated ester at a [PEG]:[antibody] molar ratio of 6:1 at room temperature, under agitation for 30 min. The extent of F(\( ab')_2 \) PEGylation was evaluated by SDS-PAGE and confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS). Gels were stained with both GelCode Blue Stain Reagent and barium iodide stain to distinguish PEGylated from unconjugated species. The reaction mixture was then dialyzed against buffer A (20 mM CH\(_3\)COONa, 5 mM NaCl, pH 4.7) overnight and was loaded on the CEC column for purification of PEG-conjugates. Free fragment was recycled and used for further PEGylation after being dialyzed against reaction buffer. The collected fractions containing the PEGylated species were gathered, concentrated and dialyzed against PBS.

PEGylation of anti-IL-17A F(\( ab')_2 \) fragment with linear PEG20-NHS was also tested. However, the reaction products were neither purified nor used in further in vitro studies. This was used to better understand the role of PEG size and shape in the reaction. pH 7.4 and 8.2 were tested. In all cases, the reaction conditions were identical to the PEGylation with PEG40-NHS, as described above.

2.8. Matrix-assisted laser desorption/ionization time-of-flight mass spectra (MALDI-TOF MS) analysis

One \( \mu \)l of a solution containing 5 mg/ml of \( \alpha \)-cyano-4-hydroxycinnamic acid (alpha-cyano MALDI matrix, Protea Biosciences, Morgantown, WV 26507, USA) in 0.1% (v/v) TFA and 50% (v/v) acetonitrile were added to 1 \( \mu \)l of each sample and mixed. One \( \mu \)l of this solution was placed on a MALDI plate Opti-TOF™ 384 Well Insert (Applied Biosystems, Foster City, USA) and dried. MS spectra were acquired with linear high mass positive acquisition method with 4800 MALDI TOF/TOF™ Analyzer spectrometer (Applied Biosystems, Foster City, USA) using a 200Hz solid state laser operating at a wavelength of \( \lambda = 355\) nm. MS spectra were obtained using a laser intensity of 6000 and 4000 laser shots by spot.

Mass calibration was performed using 1 pmol of Bovine Serum Albumin (BSA, Protea Biosciences, Morgantown, WV 26507, USA) spotted on each sample on the MALDI plate. Data were collected with the 4000 Series Explorer™ software (Applied Biosystems, Foster City, USA).

2.9. IL-17A binding activity

The antibody binding activity of the anti-IL-17A constructs was determined by a competition immunoassay. F96 MaxiSorp Nunc-Immuno™ plates were coated with 200 ng/ml mouse IL-17A cytokine (Shenandoah Biotechnology, Warwick, PA, USA) in 20 mM glycine buffered saline (GBS), pH 9.4 at 4 °C overnight and blocked with 10% fetal calf serum (FCS) in PBS at 37 °C for 1 h. Then, a mixture of constant concentration of full-length biotin-labeled anti-IL-17A and serial decreasing dilutions of unlabeled full-length anti-IL-17A or its unlabeled constructs were added and incubated at 37 °C for 2 h. More precisely, 340 ng/ml of biotin-labeled antibody and an initial concentration of 10 \( \mu \)g/ml of unlabeled antibody in 1% Bovine Serum Albumin (BSA) in PBS were used. Streptavidin–horseradish peroxidase conjugate (RPN1231VS, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was added and the plates were incubated at 37 °C for 1 h. Then, chromogenic substrate (1–Step Ultra TMB – ELISA Substrate, Thermo Fisher Scientific, Rockford, IL, USA) was added. After adequate color was achieved, the reaction was stopped by adding 2 M \( \text{H}_2\text{SO}_4 \). The peroxidase activity was detected by measuring the absorbance at 450 nm in a Multiskan Ex Microplate Photometer (Thermo Fisher Scientific, Rockford, IL, USA). A washing step was performed between each step of the method described. The binding activity was estimated by the \( K_D \) of the saturation curves, based on the antibody concentration needed for 50% of labeled antibody to be bound. The inhibition of biotin-labeled anti-IL-17A binding to IL-17A by the unlabeled modified products was compared to the inhibition observed with unlabeled full-length anti-IL-17A.

2.10. Biotin labeling of antibodies

Biotin labeling of the full-length anti-IL-17A antibody was performed using EZ-Link Sulfo-NHS-LC-Biotin reagent (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer’s procedure. Briefly, antibodies (2 mg/ml) were mixed with 10-fold molar-excess of N-hydroxysuccinimide (NHS)–activated biotin in PBS, pH 7.4 at room temperature for 1 h. Excess NHS-biotin was removed from the reaction mixture by dialysis against PBS using 6-8,000 MWCO Spectra/Port dialysis membranes (Spectrum Laboratories, Rancho Dominguez, CA, USA). Concentration of the biotin labeled antibody was measured by absorbance at 280 nm on a NanoDrop spectrophotometer 2000 (Fisher Scientific, Waltham, MA, USA) using an extinction coefficient of 1.4.

2.11. In vitro biological activity

IL-17A induces IL-6 secretion by the fibroblastic cell line C1D (Uyttenhove and Van Snick, 2006). IL-6 can be detected with IL-6-dependent 7TD1 cell line through a bioassay which was previously described (Van Snick et al., 1986). Serial dilutions of full-length anti-IL-17A antibody or its constructs were incubated with 1 ng/ml IL-17A in flat-bottom microwells for 4 h. A thousand cells of the fibroblastic cell line C1D were added. Forty-eight hours later, supernatants were collected, concentrations of induced IL-6 were determined with the 7TD1 bioassay and half maximal inhibitory concentration (IC50) was measured.

2.12. Statistical analysis

All results were expressed as standard error of the mean (SEM). Two-way analysis of variance (ANOVA) and Bonferroni posttest were performed to demonstrate statistical differences (\( P < 0.05 \)) using the software GraphPad Prism 5 (GraphPad Software, CA, USA) for Windows.
3. Results and discussion

3.1. Production of F(ab′)2 anti-IL-17A fragment

Proteolytic fragmentation of anti-IL-17A antibody using pepsin took place at pH 4 and increasing incubation times (15, 24 and 39 h) were tested. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to monitor the fragmentation. Prior to fragmentation, a single band at 150 kDa appeared on the gel which corresponded to the full-length antibody. Following incubation with pepsin, a second peak at 100 kDa was observed on the gel that represented the F(ab′)2 fragment. To have better insight on the yield of F(ab′)2 production and to purify the fragment, gel filtration chromatography (GFC) was used. Following 39 h of incubation, the highest yield was obtained (92%) without any important degradation products.

An [enzyme]-[antibody] w/w ratio of 1:20 was chosen as higher pepsin ratios could damage the binding site of the antibody molecule (Andrew and Titus, 1997). Digestion by pepsin generates F(ab′)2 fragments that encompasses the two Fab′ regions linked by the hinge region and the numerous small peptides of the Fc region (Roitt et al., 1985).

3.2. PEGylation of Fab′ anti-IL-17A fragment

PEGylation of the Fab′ antibody fragment was conducted by utilizing PEG-maleimide (PEG-mal). This is a Michael reaction that is suitable for site-specific modification of the free thiol groups. Prior to conjugation, the inter-heavy disulfide bonds of F(ab′)2 antibody fragment were reduced using 2-mercaptoethanolamine HCl (2-MEA). Several concentrations of the reducing agent were tested for the highest production yield of Fab′ fragment, while preserving the linkages between the heavy and the light chains. A 10 mM concentration of 2-MEA gave the highest yield of Fab′ (25%) and therefore was chosen for the rest of the experiments. An Ellman’s thiol assay confirmed that the Fab′ had free thiol groups and that intra- and inter-heavy disulfide bonds were not produced after reduction. Then, PEGylation was conducted by utilizing PEG-mal of 5 or 20 kDa. Factors affecting the yield and the degree of PEGylation including [PEG]:[antibody] molar ratio and temperature were tested. The reaction mainly yielded mono- and di-PEGylated products with both 5 and 20 kDA PEG-mal. Despite the different factors tested, very low yields of PEGylated Fab′ were obtained (data not shown). Consequently, this conjugation strategy was abandoned.

Poor yield of conjugates may be due to the dispersity of the PEGylated products. This can be explained by the type of antibody used in this study (mouse IgG1) that has three inter-heavy disulfide bonds (Roitt et al., 1985). Following the reduction step, three free thiol groups on the Fab′ fragment are available to react with PEG-mal hence making the single specific PEGylation hard to achieve. Having three candidate targets of modification, a mixture of mono-, di- even tri-PEGylation can theoretically be produced. Even though thiols react faster with maleimido terminal of PEG than amines, amino addition may also take place. Yet, the slightly acidic pH (6.8) used to perform the PEGylation should minimize side amine reactions.

3.3. Production and characterization of F(ab′)2-PEG anti-IL-17A fragments

PEGylation of F(ab′)2 antibody fragment via amine groups was achieved by utilizing N-hydroxysuccinimide (NHS) activated PEG-ester. Initial experiments using a 40 kDa PEG-NHS ester showed that a molar ratio of 6:1 [PEG]:[F(ab′)2] in PBS, pH 7.4 yielded 29% of mono-PEGylated F(ab′)2 at room temperature after 15 min of incubation. In the present study, emphasis was given in selectively producing the mono-PEGylated species of the anti-IL-17A mAb to maintain the maximum possible functionality. It was speculated that even if the PEG attaches in proximity to one of the two antigen binding sites of the F(ab′)2, there would be high chances that the other one remains free, retaining full functionality. Furthermore, it is well known from the literature that it is preferable to attach a reduced number of large PEGs than many small ones to maintain the antibody binding affinity (Chapman et al., 1999).

A series of factors were investigated to improve the yield of the mono-PEGylated protein production including incubation time, pH and protein concentration.

The effect of incubation time was first studied. Cation exchange chromatography (CEC) showed two peaks following PEGylation with 40 kDa PEG. The one that eluted first was the PEGylated species, whereas the second one was the F(ab′)2 fragment. The PEGylated protein eluted faster than the unmodified one due to the positive charge shielding effect of the branched PEG. After 30 min of incubation, no increase in the yield (33%) of the conjugate production was observed suggesting that a plateau was reached due to the hydrolysis of the ester. This is because the 40 kDa branched PEG that was used in this reaction has a half-life in solution shorter than 10 min. The reactivity of PEG-NHS esters toward amines (reaction rate) and hydroxide ion in water (hydrolysis rate) are profoundly correlated, as one increases so does the other. Consequently, the incubation time of 30 min was chosen for the rest of the experiments.

The effect of the pH using the 40 kDa branched PEG can be observed in Fig. 1. The left peak represents the mono- and multi-PEGylated antibody fragment, whereas the right peak shows the non-PEGylated species. The degree of F(ab′)2 PEGylation was determined by SDS-PAGE analysis of the fractions corresponding to the left peak. At both pH 7.4 and 8.2, mono-PEGylated F(ab′)2 was mainly produced. A remarkable increase in the 1xPEG40-F(ab′)2 production was yielded when increasing the pH from 7.4 to 8.2. On the other hand, PEGylation at pH 9.2 further increased the yield of the mono-PEGylated product but also led to a high number of multi-PEGylated products. This is visible as a peak shoulder on the left side of the main peak of the mono-PEGylated F(ab′)2. It was concluded that the pH was an important factor affecting the extent and degree of PEGylation when 40 kDa PEG-NHS was used. Due to the difficulty in separating different degrees of PEGylation products and the fact that the mono-PEGylated F(ab′)2 antibody fragment was the conjugate of main interest, pH 8.2 was selected as the most suitable for the reaction. The reaction occurs through a nucleophilic addition of the available electron pair of the amino group to the electrophilic carbonyl group of the succinimidyl ester. The conjugation of electron-poor PEGs to amino acid residues of proteins is highly dependent on their nucleophilicity; nucleophilic attack will only take place when the pH of the protein solution is close to or above the amine pKₐ (Koumenis et al., 2000). The most common reactive groups involved in coupling are the alpha N-terminus (pKₐ ~ 7) or epsilon amino groups (pKₐ ~ 10) of lysines. At pH 8.2 there are high chances for the epsilon amino groups of lysines to be PEGylated rather than the alpha N-terminus amino groups as they are more numerous (Koumenis et al., 2000). Lysines represent about 10 percent of amino acids in a typical protein (Fee and Van Alstine, 2006).

The effect of pH using PEG chains of different size was also investigated. This was to give more insight into the steric role of the polymer in PEGylation reactions. To achieve this, PEGylation using a linear 20 kDa PEG or a branched PEG of 40 kDa were tested at both pH 7.4 and pH 8.2. A summary of the results is shown in Fig. 2. The use of 20 kDa PEG at pH 7.4 yielded one bottom peak and one upper brown band. On the other hand, use of pH 8.2 resulted in one bottom blue and several upper brown bands. Blue bands represent the non-PEGylated F(ab′)2, whereas brown bands show
The selective single-PEGylation that is described above is probably due to the steric hindrance of the large, branched PEG that was not able to access the hidden lysines and hence had less available sites to attach. On the other hand, the shorter, linear PEG of 20 kDa was able to reach more lysine sites resulting in a higher number of PEG-conjugates. ‘Y’ shaped branched PEG chains are widely used in protein PEGylation for their increased surface shielding effect (Koumenis et al., 2000; Monfardini et al., 1995). This is useful for the effective protection against degradative enzymes.
Moreover, antigenicity is decreased due to the reduced accessibility of the PEGylated protein to antibodies (Nucci et al., 1991; Veronese, 2009).

Finally, the impact of protein concentration in the reaction mixture was studied. A five-fold increase of 1xPEG40-F(ab')2 production was observed when using a concentration of 4 mg/ml instead of 0.4 mg/ml (Fig. 3), indicating that protein concentration had a major impact on the yield of the PEGylated product. This can be explained by the fact that molecules first need to collide to allow a reaction to take place. As the concentration of reactants increases, the chances of collision also increase resulting in a higher PEGylation rate. Consequently, 4 mg/ml was employed throughout the rest of the study.

To sum up, a satisfactory yield of mono-PEGylated F(ab')2 anti-IL-17A was achieved when anti-IL-17A F(ab')2 (4 mg/ml) at pH 8.2 was allowed to react with branched PEG40-NHS activated ester at a [PEG]:[antibody] molar ratio of 6:1 at room temperature, under agitation for 30 min. This may be due to the steric hindrance effect and fast reaction/hydrolysis rate of the branched 40 kDa PEG as well as the use of the appropriate pH that likely led to epsilon-amino labeling. Upon purification, 34% unreacted, 42% mono-PEGylated and 24% multi-PEGylated F(ab')2 fragment were obtained (Fig. 4a). The SDS-PAGE analysis provided evidence to support these results (Fig. 4b). Multi-PEGylated products appeared as a separate peak in the chromatogram of Fig. 4a, in contrast to the peak shoulder observed in Fig. 1, because a slightly lower flow rate was used. Finally, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was employed in order to identify the degree of PEGylation of proteins (Basu et al., 2006; Li et al., 2007; Watson et al., 1994). The MALDI-TOF spectrum of F(ab')2 anti-IL-17A antibody fragment alone (Fig. 5a) appeared to have one dominating peak, at 97.7 kDa, equivalent to its expected molecular mass. The spectrum of 1xPEG40-F(ab')2 (Fig. 5b) showed one dominating peak, at 140.3 kDa, also equivalent to its expected molecular weight. The difference between the two species equals approximately 40 kDa. This corresponds to the molecular mass of one 40 kDa PEG chain and confirms that mono-PEGylation occurred under the conditions described above.

3.4. Binding activity of anti-IL-17A constructs

A competitive immunoassay was used to investigate whether the fragmentation and the PEG modification of the anti-IL-17A antibody affected the antigen-binding activity. For this purpose, the full-length antibody was labeled with biotin and the ability of the F(ab')2 and of the 1xPEG40-F(ab')2 to inhibit the intact biotin-labeled Ab was compared (Fig. 6). The estimated Kd of the intact mAb was 8.5 nM. The F(ab')2 fragment had essentially the same Kd value (10.5 nM) suggesting that the fragmentation did not affect the binding activity of the anti-IL-17A antibody. PEGylation of the F(ab')2 with one branched 40-kDa PEG molecule caused a 4.7-fold decrease in the binding activity (Kd = 40 nM), indicating a partial masking of the binding region by the PEG chain.

3.5. In vitro biological activity of anti-IL-17A constructs

To estimate the in vitro biological activity of the modified anti-IL-17A (F(ab')2, 1xPEG40-F(ab')2) versus the intact antibody, C11D fibroblastic cell line was used in a well-defined bioassay (Uyttenhove and Van Snick, 2006; Van Snick et al., 1986). This bioassay measures the biological competition between the C11D fibroblastic cell IL-17A receptor/binding partner and the anti-IL–17A antibody paratope for binding to IL-17A.

The effective half maximal inhibitory concentration (IC50) for intact anti-IL-17A was 320 pM. F(ab')2 had the same IC50 (324 pM) suggesting that the fragmentation had no impact on the inhibitory activity.
Fig. 4. Characterization of the reaction mixture of the PEGylation reaction under the best conditions tested: 4 mg/ml of anti-IL-17A F(ab’)2 at pH 8.2 with branched PEG40-NHS activated ester at a [PEG]:[antibody] molar ratio of 6:1 at room temperature, under agitation for 30 min. (a) Cation exchange chromatogram of reaction mixture. From left to right, the first peak represents multi-PEG40-F(ab’)2, the second corresponds to 1xPEG40-F(ab’)2 conjugate, whereas the third peak shows unmodified fragment. (b) SDS-PAGE gel of CEC fractions. Blue bands represent unconjugated F(ab’)2 and brown bands ≥250 kDa represent PEG-F(ab’)2 with one, two or three chains of PEG 40 kDa. Brown band at ~75 kDa corresponds to 40 kDa PEG alone used as a control. Fractions 17–19 contain purified 1xPEG40-F(ab’)2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
activity of the mAb. Attachment of one branched PEG of 40 kDa to the F(αb′)2 fragment produced a slight drop of about 2-fold in inhibitory activity (IC50 = 50 pm; Fig. 7).

We observed a difference between the binding activity of the PEGylated antibody fragment, tested by ELISA, and its in vitro biological activity tested in the bioassay. The PEGylated fragment had a 4.7-fold decrease in its binding activity, whereas there was only a 2-fold drop in its in vitro biological activity. Nevertheless, this should be considered with caution, as there is more intrinsic variability in in vitro biological assays than in ELISAs. There are two main factors that may affect the outcome of the in vitro assay: (i) the actual antigen binding activity of the anti-IL-17A antibody toward IL-17A and (ii) the capacity of the anti-IL-17A antibody to inhibit the binding of the IL-17A to its cell receptor. It is suggested that the decreased antigen binding activity of the PEG-conjugate could be counterbalanced by the enhancement of its inhibitory effect due to the presence of PEG. The antibody which is linked to dimer IL-17A may cause a weakness in binding affinity of IL-17A to its receptor on CD11D fibroblastic cells and this binding reduction could be enhanced due to steric hindrance of PEG. However, further investigation is needed to support this explanation.

The in vitro activity of PEGylated proteins determined by cell-based bioassays is not directly predictive of the in vivo therapeutic effect (Bailon and Won, 2009). A typical example is the PEGylated α-interferon drug Pegasis® available on the market. Pegasis® retains only 7% of the antiviral activity of the native protein but demonstrates a greatly improved performance in vivo compared with the unmodified enzyme because of improved pharmacokinetics (Veronesi and Pasut, 2005). The present results indicated that the inhibition of IL-17A by 1xPEG40-F(αb′)2 anti-IL-17A antibody was sufficiently persistent to further study their in vivo therapeutic efficacy.

4. Conclusions

PEGylation of F(αb′)2 anti-IL-17A antibody fragment with one PEG chain of 40 kDa was achieved and the mono-PEGylated product was purified. This modified molecule appeared to retain most of its binding activity and its in vitro biological activity. These results are considered to be promising and in vivo studies in mice will be carried out to further explore the efficacy of the conjugate in the treatment of bronchial hyperreactivity.

Acknowledgements

This work was supported by the WALSEO 3 programme of the Walloon Region, Belgium (Grant 816862). Rita Vanbever is Maître de Recherches of the Fonds National de la Recherche Scientifique.