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Tracking of antibody reduction fragments by capillary gel electrophoresis during the coupling to microparticles surface

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ABSTRACT

Due to their high specificity and efficiency, antibodies are ideal ligands for target-specific ultrasound contrast agents. The present study focuses on the chemical stability of antibodies during functionalisation with sulfosuccinimidyl-pyridyldithiopropionamidohexanoate (SPDP), a heterobifunctional linker, which exposes free thiol groups upon treatment with a reducing agent. Thiolated antibodies can then react with thiol-reactive group, such as maleimide present on the microbubble surface to form stable covalent complexes. The immunoglobulin structure relies on several intra- and inter-chain disulfide bridges which might be affected by reducing agents. A capillary electrophoresis-sodium dodecyl sulfate (CE-SDS) method with UV detection was applied to address the effect of the functionalisation process on the structural integrity of the antibodies and revealed that antibody disulfide bonds are prone to reduction as function of the reducing agents. Depending on the coupling conditions, various IgG fragments were identified reflecting different combinations between the light and heavy chains. Furthermore, two commonly used reducing agents, namely triscarboxyethylphosphine (TCEP) and 1,4-dithiothreitol (DTT) were compared under various preparation conditions. Results showed that reduction conditions based on DTT as a reducing agent under acidic pH were more appropriate to preserve intra- and inter-disulfide bridges of SPDP-modified antibodies.

1. Introduction

Ultrasound contrast agents (UCA) are increasingly used in clinical settings for diagnostic imaging and follow-up of patients. These agents are gas-filled (lipid or protein shelled) echogenic microbubbles that remain exclusively in the vascular compartment. The unique properties of UCA have enabled the measurement of microvascular perfusion in various organs, such as brain, heart or solid tumours [1]. Recently, microbubble constructs with targeting properties have emerged. These microbubbles can be functionalised by incorporation of target-specific ligands (peptide or antibodies) on the surface of the microbubbles to enable attachment to vascular molecular targets. Molecular imaging using targeted ultrasound contrast agents represents a new approach for the detection and monitoring of several diseases including inflammation and angiogenesis [2–5].

Two methods are mainly used for attaching targeting ligands to the surface of particles (liposome, nanoparticles, microbubbles, etc.): noncovalent or covalent attachment of the moieties [6,7]. The noncovalent approach generally relies on the use of Protein A or G, or streptavidin-modified particles. However, this approach is exclusively intended for pre-clinical use in animal models, due to strong immune response against streptavidin molecules. Covalent methods have been developed to enable direct chemical coupling of the targeting ligand to the microbubble surface. An approach based on a thioether bond is often selected as a way to prepare target-specific microbubbles. It relies on the reaction of a thiol group with a maleimide-bearing phospholipid present in the microbubble shell (e.g. DSPE-PEG-maleimide), yielding a stable thioether bond [8]. However, many targeting ligands do not contain free thiol groups. They have to be added via heterobifunctional cross-linking agents, or can be obtained by reduction of existing disulfide bonds. N-(hydroxysuccinimidyl 3-(2-pyridyldithio) propionate (SPDP) and succinimidyl-S-acetylthioacetate (SATA) are commonly used cross-linkers [9,10]. These molecules will react with active amino groups present in the targeting ligand. In a second step, the thiol group is made available by treatment with reductive agents such as DTT, TCEP (for SPDP) or hydroxylamine (for SATA).

In the development of target-specific UCA, antibodies are preferably used due to their high specificity and affinity. Coupling on the

Abbreviations: DTT, 1,4-dithiothreitol; CE-SDS, capillary electrophoresis-sodium dodecyl sulfate; LC-SPDP, long chain-succinimidyl-pyridyldithiopropionamido hexanoate; TCEP, tris(2-carboxyethyl) phosphine hydrochloride.

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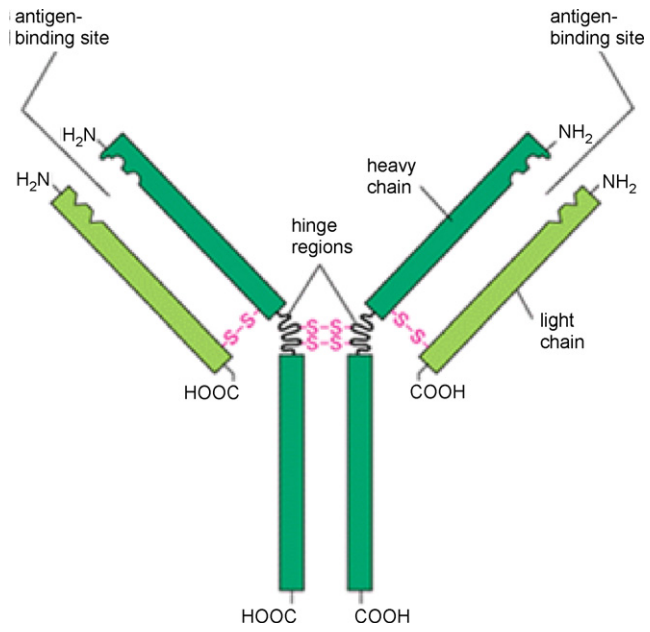

Fig. 1. Structure of a gamma immunoglobulin (IgG). Inter-chain disulfide bonds (in red) are found in the hinge region between heavy chains and in the Fab region between heavy and light chains. Intra-chain disulfide bonds, not depicted here, are found within all immunoglobulin domains of heavy and light chains.

microbubble surface can be achieved through the SPDP-maleimide chemistry. Since antibodies contain several inter- and intra-chains disulfide bonds (Fig. 1), it is important to evaluate the effect of the reduction process to generate active thiol groups on the chemical integrity of the targeting ligand. Indeed, it has been previously shown that DTT treatment of IgG damages part of the endogenous disulfide bonds [11]. Therefore, suitable analytical tools are needed to characterize and monitor possible physico-chemical alterations (e.g. reduction, oxidation, exposition of hydrophobic residues) of polypeptidic ligands [12]. Alterations may not impact on targeting efficiency but can affect their safety [13].

In the last two decades, capillary electrophoresis in the presence of sodium dodecyl sulfate (CE-SDS) has emerged as a valuable alternative to conventional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for the characterization and automatic quantitation of biomolecules and particularly monoclonal antibodies (MAbs) [14,15]. High separation efficiency, ease of operation and automation, and reduced operating costs are the main reasons of this success. Moreover, in contrast to classical SDS-PAGE slab gel technology, CE-SDS is less tedious and does not require large volumes of toxic reagents. In the CE-SDS method, SDS is bound to all proteins at a constant weight ratio and therefore the SDS protein complexes have approximately equivalent charge densities allowing an electrophoretic separation mechanism based on molecular weight differences. As a result, CE-SDS has found wide acceptance within the biopharmaceutical industry and is applied as a core analytical technology for the development, manufacturing and release of therapeutic monoclonal antibodies. Nowadays, CE-SDS methods are in routine use for in process monitoring (lot-to-lot consistency), purity assessment, detecting low level impurities and measuring size variant heterogeneity [16,17].

In the present study, CE-SDS was investigated as a rapid method to evaluate the level of fragmentation of immunoglobulin gamma (IgG) upon reduction with commonly used agents such as TCEP and DTT under various conditions. It was expected to enable selection of the best chemical approach for the functionalisation and attachment of targeting antibodies to the surface of particles avoiding the formation of fragments and preserving the structural integrity of the targeting moieties.

2. Materials and methods

2.1. Materials

Goat isotype control antibody (goat IgG) was obtained from Abcam (Cambridge, UK). Rat isotype control antibody (rat IgG) was obtained from Rockland (Gilbertsville, PA, USA). Sulfo-long chain-succinimidyl-pyridyldithiopropionamido hexanoate (sulfo-LC-SPDP), tris(2-carboxy ethyl) phosphine hydrochloride (TCEP), 1,4-dithiothreitol (DTT), IODO-GEN pre-coated iodination tubes, Zeba Desalt spin column, were purchased from Pierce (Lausanne, Switzerland). SDS-MW Analysis kit was obtained from Beckman-Coulter (Nyon, Switzerland).

2.2. Methods

2.2.1. Thiolation of antibodies using sulfo-LC-SPDP

Thiolation of antibodies was performed according to the manufacturer's recommendations. Briefly, 10 nmol (i.e. 1.5 mg) of antibody in azide-free, carrier-free and amine-free buffer were mixed with 50 nmol of sulfo-LC-SPDP reagent in 500 μL of a buffer (50 mM phosphate buffer, 150 mM NaCl, pH 7.4) and incubated for 30 min at room temperature. Thiolated antibodies were purified on a 2 mL Zeba Desalt Spin column equilibrated with 1 mL of reducing buffer (see Section 2.2.2) prior to reduction. Thiol groups were deprotected by incubation with reducing agents such as TCEP or DTT as stated below. Thiolated antibodies were finally purified on a 2 mL Zeba Desalt Spin column equilibrated with 1 mL of the relevant reaction buffer. Deprotection of thiol groups at the end of the SPDP molecule releases an equimolar amount of pyridine-2 thione (2-TP) which has a peak of absorbance at 343 nm. The number of active thiol group per antibody was calculated from this absorbance value, measured in a Lambda 35 spectrophotometer (Perkin-Elmer) immediately after reduction prior to the final purification, using the following formula: $R_{SH/Ig} = (A_{343}/8080)/[Ig] = (A_{343}/404{,}000)$, where [Ig] is the micromolar antibody concentration.

2.2.2. Reduction with TCEP or DTT

SPDP-modified antibodies were reduced with 0.5, 1, 10 or 50 mM TCEP or 10 mM DTT, for 5 min at room temperature or on ice. Reduction was carried out in the following buffers: (i) 50 mM phosphate buffer, 150 mM NaCl, pH 7.4 or (ii) 200 mM acetate buffer, 5 mM EDTA, pH 5.

2.2.3. Test of antibody integrity following chemical modification by capillary gel electrophoresis (CGE)

Antibodies have intra-chain and inter-chain disulfide bonds that are possibly sensitive to reducing agents such as TCEP or DTT. The integrity of antibodies following the reduction treatment was assessed by the CE-SDS method described below.

All CE-SDS analyses were carried out on a ProteomeLab PA 800 Protein Characterization System (Beckman-Coulter, Fullerton, CA, USA) equipped with a diode array detector. A bare fused silica capillary (BGB Analytics, Switzerland) with a total length of 30 cm (effective length 20 cm) with I.D. of 50 μm and O.D. of 375 μm was used for all CE experiments.

The CE-SDS conditions were similar to those described in the Beckman-Coulter manual for IgG Heterogeneity kit. To maintain a high reproducibility between two successive runs, the capillary was rinsed with 0.1 M NaOH, 0.1 M HCl, water, and SDS-gel buffer for 3, 1, 1 and 10 min, respectively. Both the capillary inlet and outlet were dipped twice into water to clean the viscous residual

gel buffer before sample injection. Sample injection was performed at the anode with reverse polarity using −5 kV for 20 s, then both ends of the capillary were dipped one more time to reduce sample carry over. Separation was then carried out at −15 kV for 40 min in the reverse polarity mode with 20 psi of pressure applied to both capillary ends during the electrophoretic run. A typical current of 25 μA was observed during separation. UV detection was conducted at 220 nm. Sample storage and cartridge temperatures were maintained at 25 °C.

All investigated antibodies were prepared in 100 μL of SDS sample buffer (100 mM Tris–HCl pH 9.0, 1% SDS) at a concentration of 1–2 mg/mL. Two microliters of the 10 kDa protein standard were systematically added before sample injection in the PA800 electrophoretic system.

Molecular weight estimation was obtained from the plot of log (MW) versus the relative migration time ($t_m/t_{I.S.}$) where t_m and $t_{I.S.}$ represent the migration times of a given fragment and the internal standard (10 kDa), respectively. A linear relationship was obtained with a determination coefficient higher than 0.99 (data not shown).

3. Results and discussion

3.1. Reduction of antibodies with TCEP under neutral conditions

IgG antibodies are remarkably stable proteins composed of two 50 kDa (heavy) chains and two 25 kDa (light) chains stabilized by intra- and inter-chain covalent bridges between cysteines (Fig. 1). Depending on the isotype, there are a variable number of inter-heavy chains disulfide bonds in the so-called hinge region. IgG1 and 4 have two disulfide bonds whereas IgG2 have four and IgG3 have eleven. In contrast, a single disulfide bond, at the root of the Fab domain, keeps heavy and light chains together. For some antibodies, heavy and light chains association relies solely on noncovalent interactions such as hydrogen bonding or hydrophobic avidity [18]. Inter-heavy and inter-heavy-light chains disulfides have different reduction susceptibilities depending on the species and the isotype [19–21]. Therefore it is mandatory to evaluate the chemical integrity of antibodies after treatment with reducing agents during the process of functionalisation and chemical coupling to microparticles.

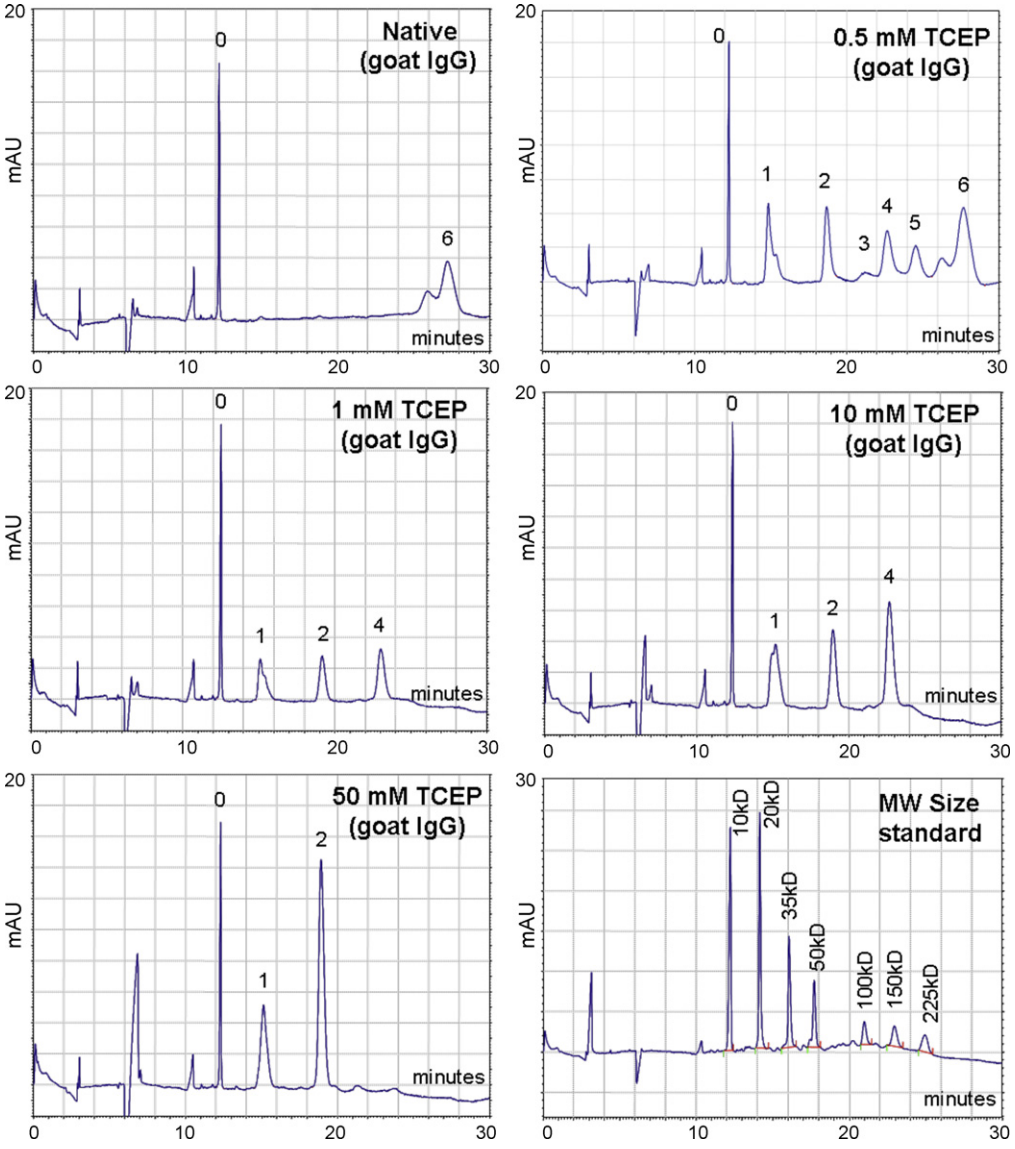

Fig. 2. Capillary gel electrophoresis analysis of control goat immunoglobulin incubated with increasing concentration of TCEP. Samples (2.5 mg/mL) were incubated with 0.5, 1, 10 or 50 mM TCEP for 5 min on ice. Peak #0 is the internal 10 kDa standard.

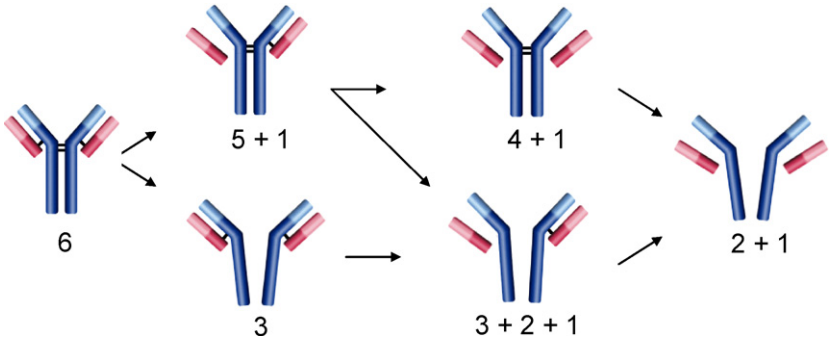

Fig. 3. Successive reductions of immunoglobulin disulfide bridges. Although there are three main routes of reduction, a mix of all intermediate species is found. The relative abundance of each species may vary from one antibody to another.

TCEP is a popular odourless reducing agent used in biochemistry and molecular biology applications. It is known to selectively and completely reduce even the most stable water-soluble alkyl disulfides over a wide pH range. In the course of our investigations on the preparation of targeted UCA using antibodies as ligands, the potency of TCEP as a reducing agent was assessed by the CE-SDS-gel technique. Thus, a goat control antibody IgG incubated with increasing concentrations of TCEP was analysed by capillary gel electrophoresis. As shown in Fig. 2, depending on the reduction conditions, various IgG subunits were detected. To accurately assign each fragment, a protein sizing ladder containing recombinant proteins of 10, 20, 35, 50, 100, 150 and 225 kDa was used to estimate the molecular weights of the observed peaks. A typical electropherogram of the molecular weight markers (MW markers) is shown in Fig. 2 (bottom right).

Large glycoproteins such as antibodies are poorly resolved by capillary gel electrophoresis. In fact, native antibodies appear as a broad split peak with high apparent molecular weight (peak #6, top left electropherogram). Peak #0 corresponds to the 10 kDa internal standard used to assign fragment identification. At the lowest TCEP concentration (i.e. 0.5 mM), beside intact IgG, various IgG fragments are detected corresponding to different combinations between heavy and light chains (top right electropherogram). Fig. 3 shows various possible fragments generated following disulfide bonds reduction. The estimated molecular weights of the corresponding fragments as obtained with the help of MW markers are reported in Table 1. At concentrations between 1 and 10 mM of TCEP, only single light chains (peak #1), single heavy chains (peak #2) and heavy chain dimers (peak #4) are present showing that disulfide bridges between heavy and light chains are weaker. At 50 mM, goat IgG is fully reduced to heavy and light chains (peaks #2 and #1, respectively). Similar results were observed for a rat control IgG antibody (data not shown).

Table 1
Estimated molecular weights and identification of the different reduction fragments on the electropherograms.

Peak #	Migration time (min)	Calculated MW (kDa)	Ig chain
0	12.3	10	Not applicable
1	14.9	24	Light
2	18.7	55	Heavy
3	21.3	79	Heavy + light
4	22.7	110	2 Heavy
5	24.5	134	2 Heavy + light
6	27.5	158	2 Heavy + 2 light

Since these first investigations were performed on native unmodified antibody, additional experiments were carried out to confirm that a functionalised antibody would show a similar behaviour. Thus, goat control IgG antibody functionalised with LC-sulfo-SPDP at a 6–1 molar ratio was incubated with a low concentration of TCEP (0.5 mM) either at room temperature or on ice and analysed by capillary gel electrophoresis (Fig. 4). Goat IgG was sensitive to TCEP as several reduction fragments were detected even under the mildest conditions (low temperature). Reduction of goat IgG is almost complete after 10 min at room temperature generating mainly light (peak #1) and heavy chains (peak #2) as well as heavy chain dimers (peak #4). Even if the integrity of the control antibodies used herein seemed challenged by a mild reducing treatment, functional tests involving target recognition (e.g. ELISA) must be carried out to convey a biological significance. This test was performed with a target-specific antibody (namely rat anti-mouse

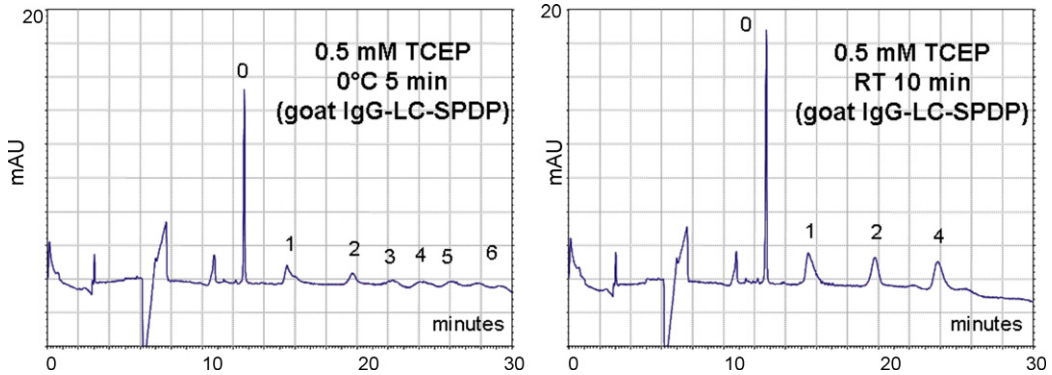

Fig. 4. Capillary gel electrophoresis analysis of immunoglobulins functionalised with SPDP and incubated with 0.5 mM of TCEP. Goat Ig-SPDP (top lane) breaks down in all possible intermediates at both low temperature and room temperature. Peak #0 is the internal 10 kDa standard. See Fig. 3 and Table 1 for the explanation of the peak numbers.

P-Selectin antibody, RB40.34 clone IgG1), for which TCEP treatment decreased the affinity of the antibody (as assessed by ELISA, data not shown). This result shows that these conditions are not favourable to keep the antibody intact and prepare target-specific microparticles for example.

3.2. Reduction of antibodies with TCEP and DTT under acidic and neutral conditions

DTT is another reducing agent commonly used in various biochemical and biological applications. It is used to reduce disul-

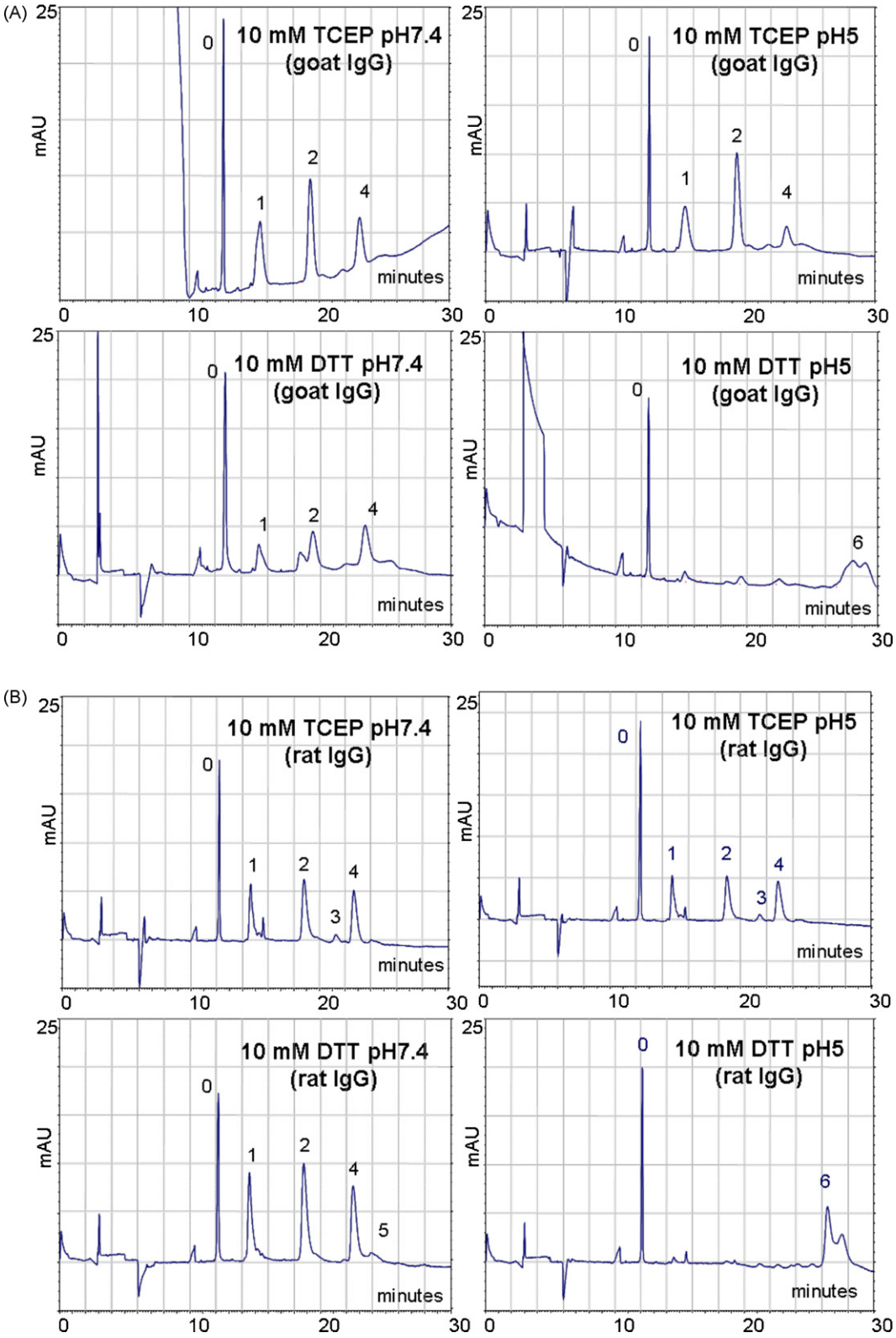

Fig. 5. Capillary gel electrophoresis of control antibody subjected to reduction: (A) goat control IgG and (B) rat control IgG. Antibodies were reduced either in acetate buffer pH 5 (right column) or PBS pH 7.4 (left column). Peak #0 is the internal 10 kDa standard.

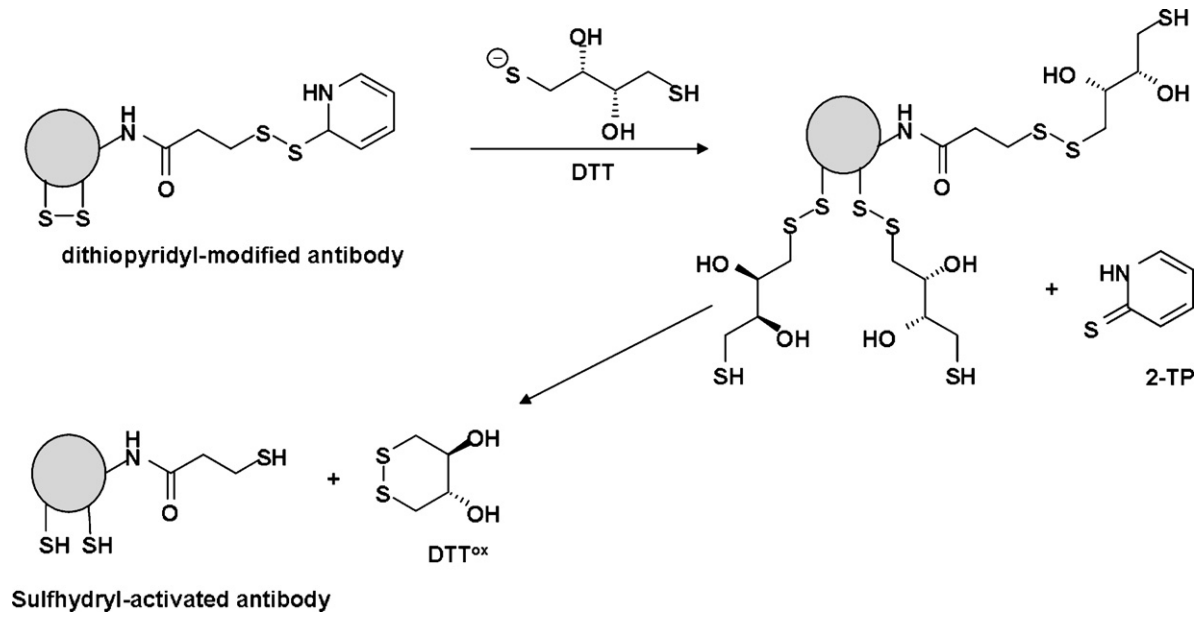

Fig. 6. Reduction of antibody with DTT in neutral conditions. At neutral or alkaline pH, all disulfides are subjected to nucleophilic attack from thiolate ions, leading to a fully reduced thiolated antibody and the release of the cyclic oxidised form of DTT (DTT^{ox}).

fide bonds of proteins and peptides and prevent intramolecular and intermolecular disulfide bonds formation between cysteine residues of proteins. In order to set up an alternative reduction protocol that would preserve antibody disulfide bonds, goat and rat IgG antibodies were reduced with TCEP or DTT either in PBS at pH 7.4 or in acetate buffer at pH 5 prior to CE-SDS analysis (Fig. 5A and B). As explained earlier, the size and nature of the different peaks of the electropherogram are listed in Table 1 and Fig. 3. With TCEP, three peaks corresponding to heavy (peak #2) and light (peak #1) chains as well as heavy chain dimers (peak #4) were observed whatever the pH of the reaction mix (Fig. 5A top lane). With DTT, these peaks were only observed in PBS whereas in acetate buffer only a single peak, corresponding to the native antibody was present (Fig. 5A bottom lane). The same result was obtained with a rat control antibody (IgG, Fig. 5B) indicating that this behaviour is not species-specific. These results indicate that in contrast to TCEP, antibody reduction by DTT is completely abolished under acidic pH conditions. Under these acidic conditions, a quantitative release of the pyridine dithione group was measured proving that these conditions preserve the integrity of the antibody and enable deprotection of the thiol groups (data not shown).

TCEP is a very efficient thiol-free reducing agent with a much greater redox potential than DTT (so lower concentrations are generally required) that can react over a wide pH range [22]. Indeed

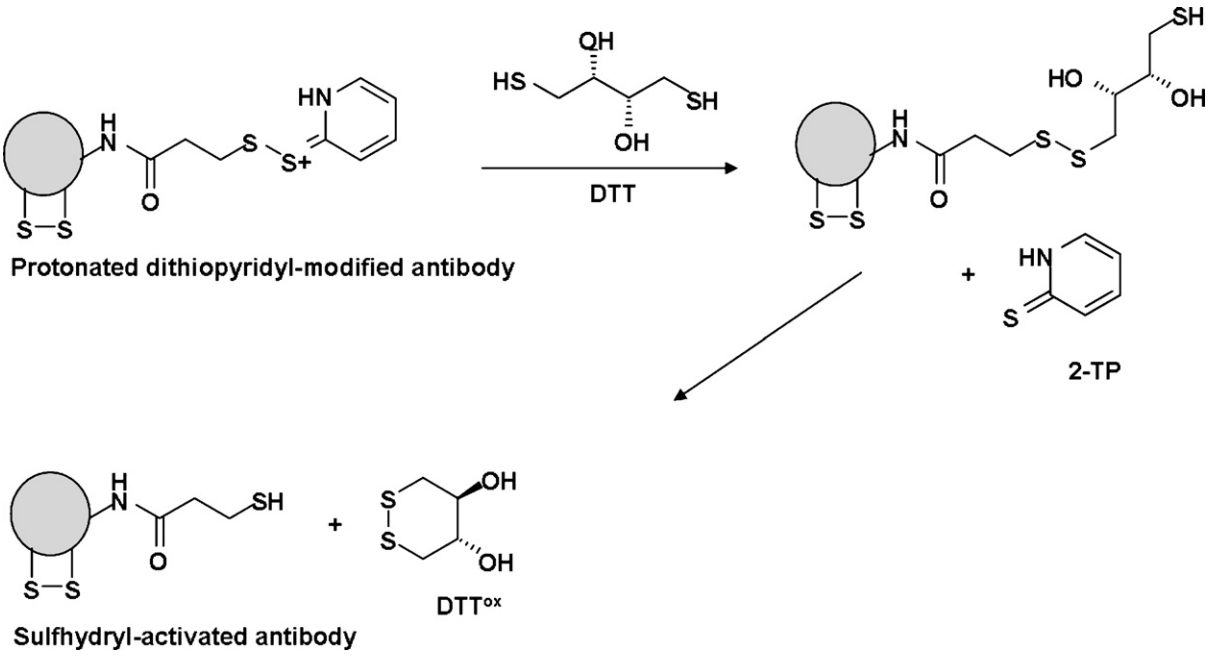

Fig. 7. Reduction of antibody with DTT in acidic conditions. The thiopyridine ring is protonated at low pH thus increasing by about a thousand-fold the electrophilicity of the nearby sulfur. Only the reactive sulfur from the thiopyridine ring readily reacts with protonated DTT, leading to an intact thiolated antibody and the release of the cyclic oxidised form of DTT (DTT^{ox}).

TCEP reactivity is not affected in acidic environment [23]. DTT as opposed, is considered as inefficient below neutrality because disulfide interchange between cysteine disulfide and DTT is subject to acid–base equilibrium (Figs. 6 and 7) [24]. As shown in Fig. 6, at neutrality all disulfide bonds are attacked by highly reactive thiolate ions, leading to a fully reduced antibody. By acidifying the reaction mix, the proportion of thiol available as thiolate on the DTT molecule decreased, and therefore disulfide interchange was reduced [25]. On the other hand this drop in thiolate was largely compensated by the enhanced reactivity of the SPDP disulfide when the pyridine ring is protonated (Fig. 7) [26]. Specific reduction at low pH of dithiopyridine by DTT without alteration of the protein disulfides has been described in several publications [27–29]. In addition, this will also reduce disulfide attack by 2-thiopyridine released from SPDP thus contributing to preserve antibody integrity.

4. Conclusions

This study reports the use of capillary gel electrophoresis to evaluate the chemical integrity of IgG during functionalisation with SPDP prior to chemical coupling to ultrasound contrast agent microbubbles for targeting purposes. Besides apparent molecular weight estimation, CE-SDS was found to be a simple and powerful technique to monitor the IgG structural integrity under various reduction conditions (reducing agent nature and concentration, reaction time and pH as well as temperature). It was particularly shown that the use of reducing agents such as TCEP or DTT during the deprotection step of the antibody thiolation protocol leads to a partial reduction of intra- and inter-chain disulfide bonds at neutral pH. Finally, it was demonstrated that disulfide interchange can be orientated towards specific chemical groups by adjusting the pH of the reaction. Thus, it was possible under acidic conditions (i.e. pH 5) to keep intact the chemical structure of antibodies (from rat or goat origin) and at the same time perform the successful deprotection to generate reactive thiol groups.

The reduction of control antibodies in all intermediate isoforms down to single heavy and light chains was followed with great accuracy and provided valuable read-outs following chemical modification of antibodies. Results indicate that CE-SDS is useful to optimize the chemical process of pyridyl dithio deprotection and thus to achieve efficient chemical coupling onto microbubbles. This is a prerequisite for the preparation of efficient targeted microbubbles suitable for various molecular imaging applications.

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