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RESEARCH PAPER

Liquid Formulations for Stabilizing IgMs During Physical Stress and Long-Term Storage

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ABSTRACT

Purpose To develop a liquid formulation for IgMs to survive physical stress and storage.

Methods Stabilizing formulations for 8 monoclonal immunoglobulin (IgMs) were found using differential scanning calorimetry (DSC). In these formulations, the IgMs were subjected to stress and storage and analyzed by size exclusion chromatography and fluorescence activated cell sorting. Structure was analyzed using small-angle X-ray scattering (SAXS).

Results The highest conformational stability was found near the isoelectric point and further enhanced by addition of sorbitol, sucrose and glycine. For 2 IgMs, the pH optimum for conformational and storage stability did not correspond. Lowering the pH led to the desired storage stability. Optimized formulations prevented aggregation and fragmentation from shear stress, freeze-thaw cycles, accelerated storage and real time storage at 4°C and −20°C for 12 months. Optimized formulations also preserved immunoreactivity for 12 months. SAXS indicated that IgM in stabilizing conditions was closer to the structural IgM model (2RCJ) and less susceptible for aggregation.

Conclusions A long-term stabilizing formulation for 8 IgMs was found comprising 20% sorbitol and 1 M glycine at pH 5.0–5.5 which may have broad utility for other IgMs. Formulation development using DSC and accelerated storage was evaluated in this study and may be used for other proteins.

KEY WORDS conformational stability · DSC · formulation · IgM · protein aggregation

INTRODUCTION

Monoclonal immunoglobulins M (IgMs) are serious candidates for next generation antibody therapeutics. So far all approved therapeutic antibodies are IgGs (1), but the potential of monoclonal IgMs as future therapeutics with possible applications in infectious disease or cancer has already been clearly demonstrated (2–4). A major challenge is to produce high amounts of pure and active IgMs for laboratory and clinical trials and to stabilize the IgMs for long term storage. However, little is known about the formulation and stabilization of IgMs. Only a few publications report the influence of excipients on IgM stability (5,6).

Proteins like IgMs are prone to degradation by a variety of pathways which can be physical (e.g. aggregation, denaturation), chemical (e.g. deamidation, oxidation, deglycosylation, fragmentation) and biological like proteolysis (7,8). Those alterations may impact protein quality, efficacy and safety. For example, antibody aggregates present a greater immunogenicity than monomers and can cause serious side effects (9,10).

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A variety of excipients increase the stability of different proteins during freeze-thaw cycles, storage at elevated temperature and real time storage (11–15). The main mechanism of stabilization is *via* preferentially exclusion of a solute from the protein surface which leads to a layer of excess water surrounding the protein (16,17). Thus, the protein is forced to minimize its surface area and forms a more compact state. In such a way unfolding becomes thermodynamically less favorable than folding, thus leading to stabilization (16,17). Excipients that are preferentially excluded from a protein surface include polyols like sucrose (16), sorbitol (17) and glycerol (18), certain salts (19), certain amino acids (20) and polymers like polyethylene glycol (PEG) (14). Surfactants protect proteins during freeze-thaw cycles and shear stress *via* a different mechanism. They prevent proteins from air-liquid or ice-liquid interphase induced adsorption, denaturation and aggregation by occupying this interface competitively (13,21).

Optimization of formulations by real time storage is time consuming and expensive, so faster alternative methods for prescreening are preferred such as differential scanning calorimetry (DSC). Using this method, the indicator for thermal or conformational stability is the temperature midpoint of transition (T_m) where 50% of the protein is folded and 50% is unfolded. Multidomain proteins like IgMs can show more than one thermal transition (22). For IgGs, it was shown that the 2–3 found transitions correspond to different structural domains or specific areas, such as Fab or Fc region, or CH1 and CH2 domains of Fc (23,24). Formulations are usually optimized based on the transition of the least stable domain (T_m1). In the ideal case, proteins with high thermal stability show increased storage stability (11,12), but in some studies high thermal stability did not lead to low aggregation rate (25,26). Thermal stability and aggregation rate correlate only if the mechanism of aggregation is related to protein unfolding. An increase of the T_m leads to a shift of the equilibrium to the native state leading to reduction of unfolded protein population and thus reduction of the aggregation from unfolded proteins. Excipients or conditions that increase the T_m can in parallel enhance self-association of proteins in unfolded and native state (27).

Furthermore, accelerated storage studies at elevated temperatures are often performed for fast screening of formulations (28). Those studies do not allow a full prediction due to several assumptions in the Arrhenius law. Furthermore, it was shown recently that the mechanism of aggregation is temperature dependent, so that aggregation rate at elevated temperatures does not always correlate to the rate at low temperatures (29). Thus real time storage studies are still necessary to test shelf life of proteins.

At Bioprocessing Technology Institute, mouse monoclonal IgMs selectively targeting surface markers on undifferentiated human embryonic stem cells (hESC) were generated (30). One IgM (mAb 84) did not only bind but also kill hESC *via* oncosis (31). Liquid formulations for 8 of these anti-hESC IgMs were optimized using DSC with the aim of providing stability during mixing, repeated freeze-thawing cycles, short term storage at 4°C and long term storage at −20°C. This study may contribute to the fundamental knowledge of IgM stability and formulation, and shows a method for optimization of long-term stabilizing IgM formulations using DSC, stress and accelerated storage studies.

MATERIALS AND METHODS

Antibodies

Monoclonal mouse IgMs (mAb 5, 14, 63, 84, 85, 95, 432 and 529) were produced in hybridomas and purified using a two-step purification comprising PEG precipitation and anion exchange chromatography as previously described (32).

Chemicals

Glycine, sodium acetate, sodium chloride, sodium dihydrogen phosphate monohydrate, di-sodium hydrogen phosphate and potassium sulfate were purchased from Merck (Darmstadt, Germany). HEPES (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid), sorbitol, glycerol, Tween 80, arginine and histidine were purchased from Sigma (St. Louis, MO, USA). Sucrose was obtained from AnalaR (Princeton, NJ, USA). All buffers were filtered using 0.2 µm nitrocellulose membranes prior to usage (Millipore, Carrigtwohill, Ireland). Nap-5 columns (GE Healthcare, Uppsala, Sweden) were used to exchange the IgMs into the buffers tested.

Differential Scanning Calorimetry

DSC measurements were performed using VP-Capillary DSC system (Microcal Inc, Northampton, MA, USA) which was equipped with tantalum cells with an active volume of 130 µl. The samples with a concentration of 1 mg/mL were scanned from 30 to 100°C using a scan rate of 60°C per hour. The corresponding buffer was used as a reference. Data were analyzed using Origin 7.0 software (OriginLab Corporation, Northampton, MA, USA). Thermograms were corrected by subtraction of buffer blank scans and normalization to the protein concentration. The transition curves were fitted using a non-2 state model to determine the T_m.

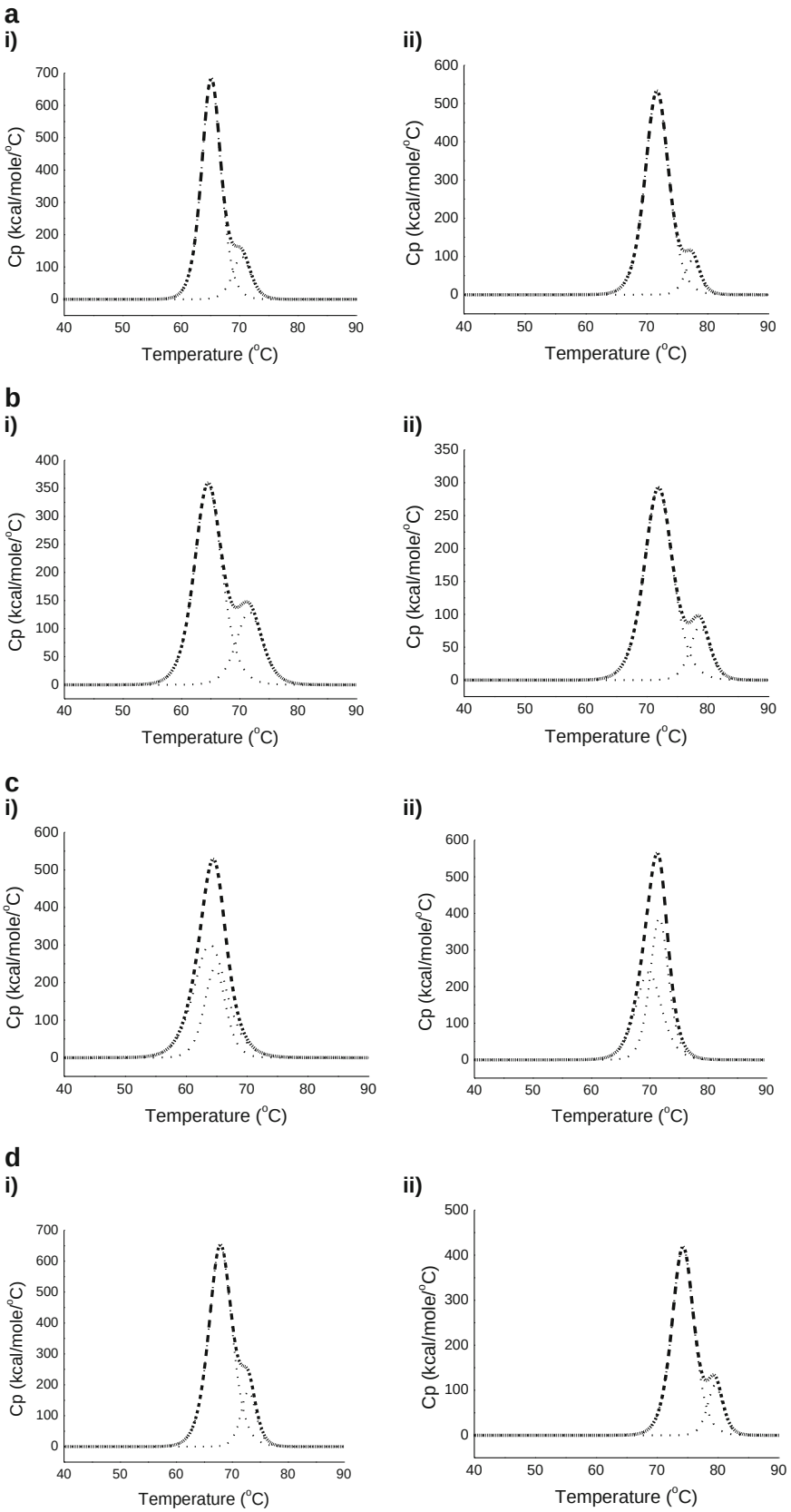

Fig. 1 Thermogram of (**a**) mAb 5, (**b**) mAb 14, (**c**) mAb 63, (**d**) mAb 84, (**e**) mAb 85, (**f**) mAb 95, (**g**) mAb 432, (**h**) mAb 529 (*i*) at their optimal pH without excipients and (*ii*) in the optimized buffer including 1 M glycine, 20% sorbitol at optimal pH (see Table II). The *dashed curve* shows the overall unfolding curve. The *dotted curves* show the separated unfolding peaks after deconvolution.

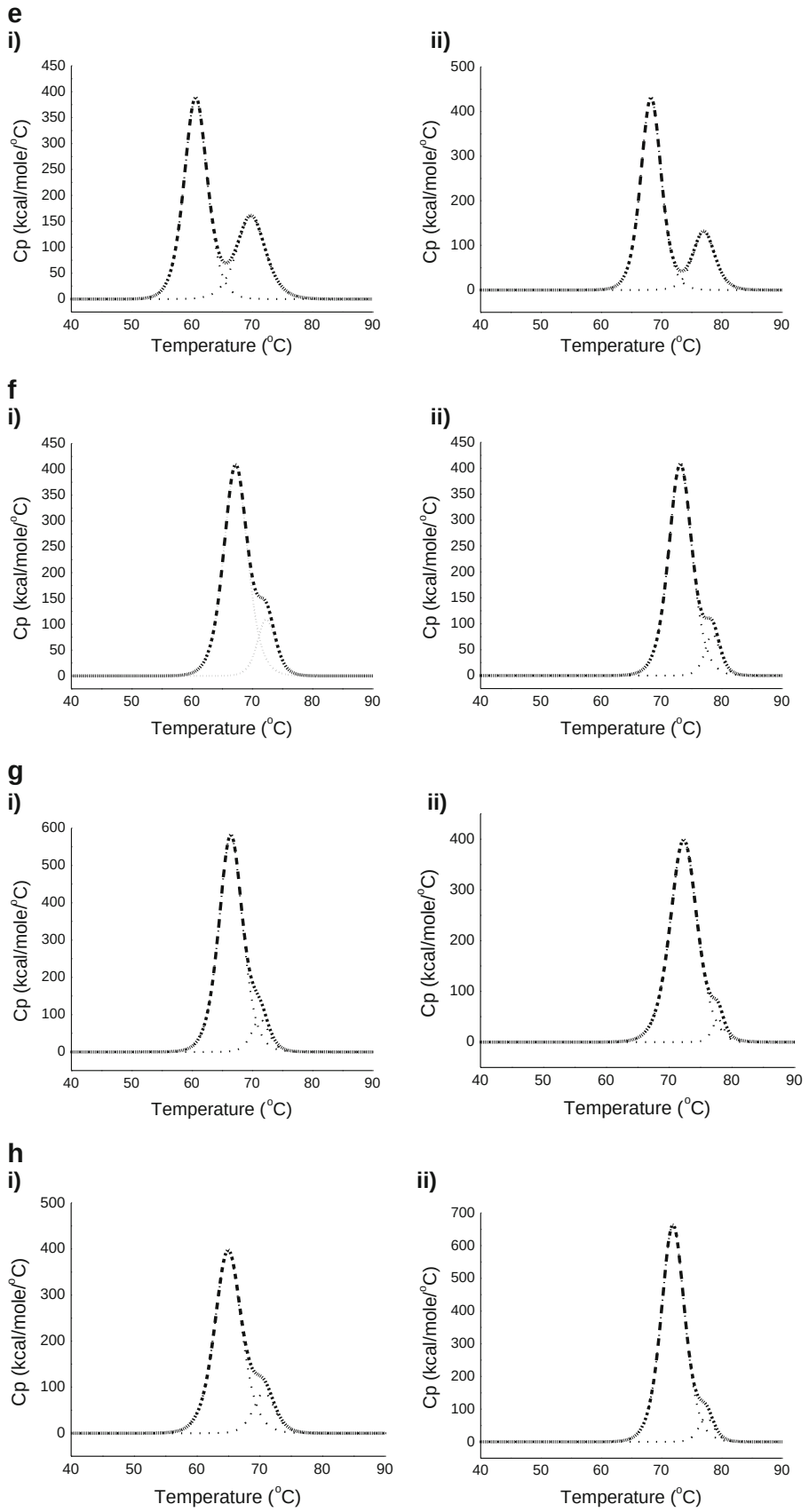

Fig. I (continued)

Table I pI of IgMs as Previously Determined by Isoelectric Focusing (40) and T_ms of the IgMs Depending on the pH of the Buffer: The T_m of the First, Second and Third Transition are Listed in the Table Below. NS Means that the IgM was not Soluble at the Indicated pH

	pI		pH 5	pH 5.5	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0
mAb 5	~5.5	T_{m1}	63.8 ± 0.3	65.0 ± 0.4	64.7 ± 0.2	64.4 ± 0.1	63.7 ± 0.1	63.2 ± 0.1	63.0 ± 0.1
		T_{m2}	64.9 ± 0.4	70.4 ± 0.0	–	70.0 ± 0.2	71.7 ± 0.1	72.0 ± 0.1	72.0 ± 0.2
mAb 14	~7.7	T_{m1}	64.6 ± 0.0	NS	NS	NS	NS	NS	NS
		T_{m2}	71.6 ± 0.1	NS	NS	NS	NS	NS	NS
mAb 63	~7.6	T_{m1}	64.0 ± 0.3	NS	NS	NS	NS	NS	63.4 ± 0.0
		T_{m2}	64.9 ± 0.1	NS	NS	NS	NS	NS	72.4 ± 0.0
mAb 84	6.5–7.5	T_{m1}	65.5 ± 0.0	66.1 ± 0.4	66.9 ± 0.3	NS	67.8 ± 0.2	68.0 ± 0.0	67.9 ± 0.0
		T_{m2}	67.4 ± 0.1	68.2 ± 0.1	–	–	72.7 ± 0.3	72.9 ± 0.0	72.5 ± 0.2
mAb 85	~6.0	T_{m1}	59.6 ± 0.2	60.7 ± 0.0	59.5 ± 0.5	59.8 ± 0.5	60.4 ± 0.1	60.2 ± 0.1	60.2 ± 0.0
		T_{m2}	67.1 ± 0.2	69.8 ± 0.2	68.2 ± 0.3	70.1 ± 0.2	63.3 ± 0.0	68.7 ± 0.6	68.3 ± 0.4
		T_{m3}	–	–	–	–	72.6 ± 0.7	73.0 ± 0.2	73.1 ± 0.0
mAb 95	6.0–7.0	T_{m1}	64.5 ± 0.3	66.5 ± 0.1	66.3 ± 0.1	NS	67.2 ± 0.1	66.7 ± 0.1	66.9 ± 0.1
		T_{m2}	–	–	–	–	72.7 ± 0.0	72.5 ± 0.6	72.6 ± 0.4
mAb 432	5.5–6.5	T_{m1}	63.8 ± 0.1	66.5 ± 0.1	66.0 ± 0.2	66.5 ± 0.5	66.1 ± 0.1	66.1 ± 0.1	66.2 ± 0.0
		T_{m2}	65.8 ± 0.1	71.2 ± 0.5	–	–	72.6 ± 0.1	72.7 ± 0.1	72.8 ± 0.2
mAb 529	6.0–7.0	T_{m1}	61.4 ± 0.7	65.1 ± 0.1	NS	NS	64.6 ± 0.1	64.8 ± 0.1	65.0 ± 0.0
		T_{m2}	64.1 ± 0.1	70.8 ± 0.0	–	–	72.0 ± 0.1	72.3 ± 0.1	72.4 ± 0.0

Analytical Size Exclusion Chromatography (SEC) with Ultraviolet (UV) and Static Light Scattering (SLS) Detection

SEC separations of aggregates, monomers and fragments were conducted on a Shimadzu LC-10Avp series high pressure liquid chromatographic (HPLC) system (Shimadzu Corporation, Kyoto, Japan) connected to a TSKgel G4000SW_{XL} column (7.8 mm × 30 cm; Tosoh, Tokyo, Japan). A running buffer of 0.2 M sodium phosphate, 0.1 M potassium sulphate, pH 6.0 was used at a flow rate of 0.6 mL/min. For SEC-UV, the injection volume of the filtered sample (0.2 μm filter, Millipore, Billerica, MA, USA) at a concentration of 1 mg/mL was 35 μL. The elution profile was detected by UV absorbance at 280 nm. Data were analyzed using Shimadzu Class-VP software (Version 6.14 SP2). For SEC-SLS, the injected volume was 100 μL of a 1 mg/mL sample. A static and quasi-elastic light scattering detector (Dawn 8 and QELS) and refractive index detector (Optilab-rEx) from Wyatt Technology Corporation (Santa Barbara, CA, USA) were used. Data were analyzed using Wyatt's Astra software (Version 5.3.4.14).

Native Polyacrylamide Gel Electrophoresis (PAGE)

Native PAGE was performed using 4% Novex® Tris-Glycine gels according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). In brief, the samples were diluted with Native Sample Buffer to a concentration of 50 μg/mL, loaded onto precast gels and separated using Native Running buffer and a Novex XCell SureLock Mini-Cell PAGE system with a voltage set to 125 V for 90 min. Silver staining of the gel was performed using SilverQuest Silver Staining Kit according to the manufacturer's protocol (Invitrogen).

Immunoreactivity of IgMs by FACS Analysis

hESC (HES-3, ES Cell International, Singapore) were cultured as described previously (41). Single-cell suspensions were harvested and suspended in 1% bovine serum albumin/PBS. 100μL of hESC (2*105 cells) was incubated with each IgM (10 μl of 1 mg/ml) at 4°C for 30 min. Then, the cells were incubated with the secondary antibody, a fluorescein isothiocyanate conjugated polyclonal rabbit anti-mouse IgG (1:500, DAKO, Glostrup, Denmark) for 15 min at 4°C. Cells were washed twice and resuspended in 1% BSA/PBS complemented with 1.25 mg/mL of propidium iodide for analysis using flow cytometry (Becton Dickinson (BD) FACSCalibur, BD Biosciences, San Diego, CA, USA) to determine binding and cytotoxicity of the IgMs.

Optimization of the Formulation pH Using DSC

Different formulation pH values were screened varying from pH 5.0 to 8.0 using 50 mM acetate buffer for pH 5.0 and 5.5, 50 mM histidine for pH 6.0 and 6.5 and 50 mM HEPES buffer for pH 7.0, 7.5, 8.0. The formulation pH was optimized for each IgM based on the highest value for T_m1. If the pH influence was not significantly different over a certain pH range (like for mAb 85, 432 and 529 between pH 5.5 and 8.0), the lowest possible pH was chosen because of a lower chemical degradation rate at low pH.

Optimization of the Excipient Concentration Using DSC and Design of Experiments (DOE) Software

The excipient concentration was optimized using the Design of Experiments (DOE) software MODDE 9.0 (Umetrics, Umea, Sweden) in 2 steps: DOE Screening followed by DOE optimization.

DOE Screening

The effect of 8 excipients (factors) on T_m1 (response) at optimized pH was tested using a fractional factorial design which is a balanced subset of corner experiments drawn from the corresponding full factorial design (33). Upper and lower limits of the concentration were chosen according to

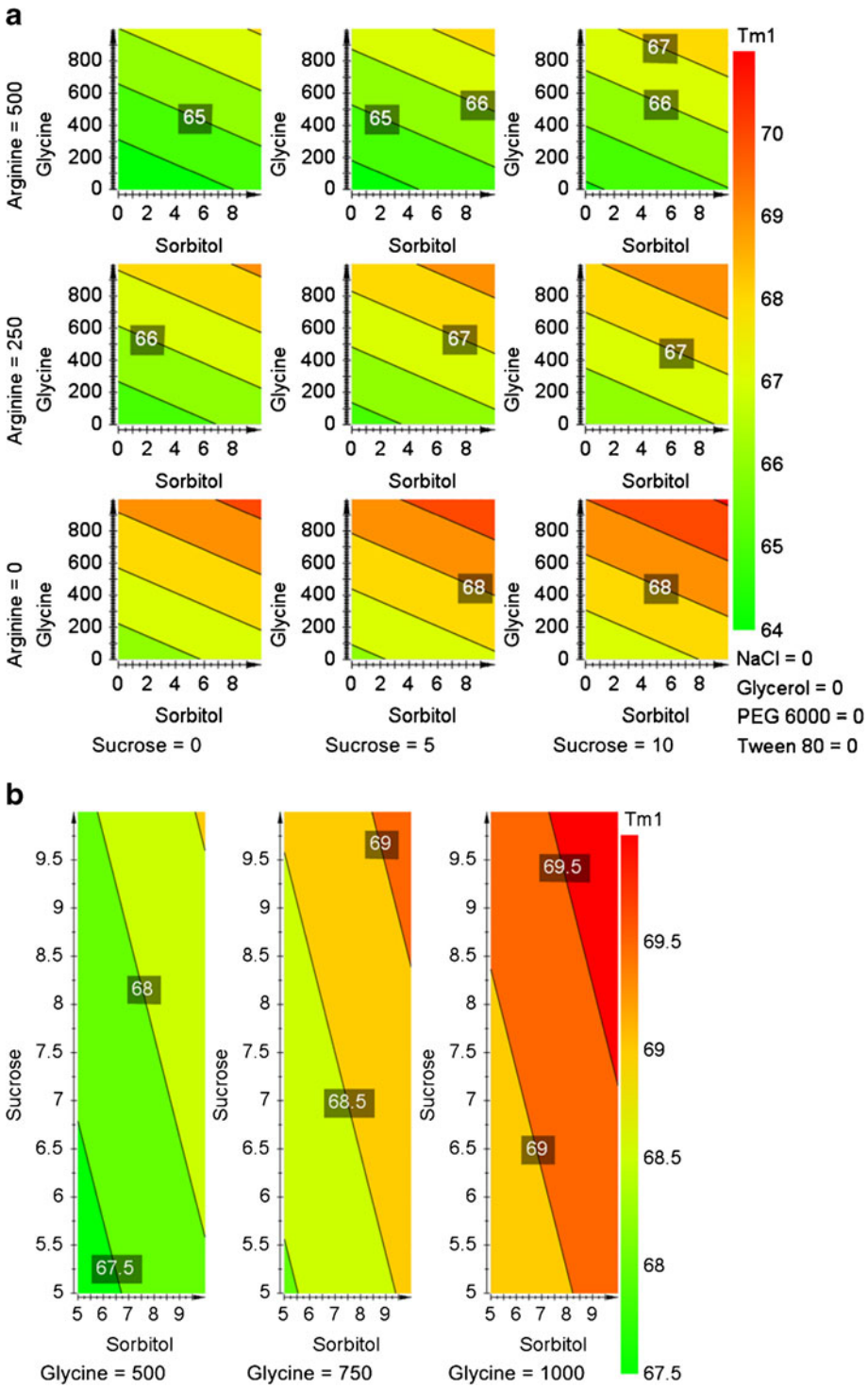

Fig. 2 Optimization of the excipient concentration for mAb 5: (**a**) DOE Screening for 8 excipients and (**b**) DOE optimization for 3 excipients: coefficient blot and response contour blot. The not significant factors were set to zero for the response contour blot.

previous literature reports (11,34,35) and prestudies (data not shown): sorbitol (0–10%), sodium chloride (0–200 mM), sucrose (0–10%), arginine (0–500 mM), glycerol (0–5%), glycine (0–1,000 mM), PEG 6000 (0–0.5%) and Tween 80 (0–0.1%) (Table 1, Supplementary Material).

DOE Optimization

The factors which have a significant stabilizing influence in the initial screening were further analyzed using a central composite face-centered design. This is an extension of the 2-level full factorial design including additional axial points located on the faces of the design cube (33). All factors were varied at three levels (high, middle, and low) (Table 2, Supplementary Material) to accurately develop a quadratic model including any curvature and determine potential interactions (33).

Stress Studies, Accelerated and Real-Time Storage

IgMs (1 mg/ml) were exposed to shear stress by mixing with an end-over-end mixer (SB3–Rotator, Stuart, UK) at 30 rpm for 24 h at 4°C. Freeze-thaw studies were performed in 5 cycles where samples were frozen at −20°C and thawed at room temperature. Accelerated storage studies were performed at 37°C for up to 4 weeks. In parallel, the IgMs were stored at 4°C and −20°C for up to 12 months. After the exposure to stresses or storage, the samples were analyzed by SEC-UV to determine aggregate or fragment formations. The initial fragment and aggregate content was deducted from the values of the stressed or stored samples.

Comparison of the Stability in the Optimized Formulation *Versus* PBS

The stability of mAb 84, 85 and 529 (as examples) in optimized formulation was compared to the stability in PBS under accelerated storage studies at 37°C for up to 6 months. The fragment and aggregate formation and the molecular size were determined using HPLC-SLS.

Small-Angle X-ray Scattering (SAXS)

SAXS measurements of mAb 529 were performed in two different buffers: 50 mM HEPES, pH 7.5 and the optimized formulation (20% sorbitol, 1 M glycine, pH 5.5). Scattering experiments were performed at beamline 12-3-1 at the Advanced Light Source (ALS), Lawrence Berkeley National Laboratory, USA. Data were collected at beamline 12-3-1 at the ALS with three exposures: 0.5, 1 and 6 s (36,37). The reconstruction of the structural model has been performed according to reference using reversed Monte Carlo simulation (38).

Statistics

All experiments were performed in triplicate in independent experiments. Data are expressed as mean ± standard error of the mean.

RESULTS

Finding Optimal Storage pH Using DSC

The DSC profile of the antibodies shows 2 separated to partially overlapping peaks (Fig. 1i, Table I). For mAb 85 even 3 unfolding peaks were found in buffers at pH 7–8. The pH optimum of the least stable domain of mAb 5, 85, 432 and 529 was at pH 5.5, the pH optimum of mAb 14 and 63 at pH 5.0 and the pH optimum of mAb 84 and mAb 95 at pH 7.5 and 7.0, respectively. The pH optima of the more stable domain were between 7.0 and 8.0. The

Table II Comparison of the Influence of Sucrose and Sorbitol on the Thermal Stability in the Buffer Containing 1 M Glycine and at Optimized pH. The T_ms of mAb 84 and 95 in 1 M Glycine and 20% Sorbitol at pH 5.5 are also Listed. NA… Means not Analyzed

	pH	10% sorbitol, 10% sucrose		20% sorbitol	
		T_m1	T_m2	T_m1	T_m2
mAb 5	5.5	69.1 ± 0.1	76.3 ± 0.4	71.9 ± 0.1	77.0 ± 0.5
mAb 14	5.0	68.7 ± 0.4	75.3 ± 0.5	72.0 ± 0.3	77.8 ± 1.0
mAb 63	5.0	66.1 ± 0.5	68.6 ± 0.5	69.8 ± 0.1	71.7 ± 0.1
mAb 84	7.5	73.3 ± 0.0	78.7 ± 0.0	73.7 ± 0.0	79.1 ± 0.0
mAb 84	5.5	N.A.	N.A.	69.5 ± 0.1	72.1 ± 0.5
mAb 85	5.5	65.5 ± 0.4	75.0 ± 0.3	68.4 ± 0.2	76.9 ± 0.2
mAb 95	7.0	70.5 ± 0.1	76.0 ± 0.4	72.9 ± 0.6	78.0 ± 0.7
mAb 95	5.5	N.A.	N.A.	71.5 ± 0.1	–
mAb 432	5.5	70.2 ± 0.4	75.6 ± 0.4	72.4 ± 0.0	77.6 ± 0.4
mAb 529	5.5	69.0 ± 0.2	74.8 ± 0.4	72.1 ± 0.4	77.1 ± 0.5

adequate pH leading to the highest T_m1 was chosen for further buffer optimizations.

DOE Screening of Excipients

For all 8 IgMs, the same trend was seen (Fig. 2a; Figure 1, Supplementary Material): Sorbitol, sucrose and glycine significantly increased the T_m1 and arginine decreased the T_m1. Up to 200 mM NaCl, 5% glycerol, 0.5% PEG 6000 and 0.1% Tween 80 did not significantly influence the T_m1. The difference between the least stabilizing formulation and the most stabilizing formulation was between 5 and 6°C. The influence of the excipients on T_m2 is summarized in Table 3 (Supplementary Material).

DOE Optimization of Excipients

Using DOE optimization, the optimal point for the highest T_m1 of all IgMs was found to be at the highest excipient concentration 10% sorbitol, 10% sucrose and 1 M glycine (Fig. 2b; Figure 1, Supplementary Material). Glycine alone was sufficient to increase T_m2 of mAb 5. Sorbitol, sucrose and glycine increased T_m2 of mAb14, mAb 84, mAb 85, mAb 95, mAb 432 and mAb 529.

According to the prediction function in the DOE software, 20% sorbitol leads to a higher T_m1 than a mixture of 10% sorbitol and 10% sucrose. Measurements confirmed that formulations with 20% sorbitol led to 2–3°C higher T_m1s (Table II). Consequently, the buffers that were chosen for accelerated and real time stability studies contained 20% sorbitol and 1 M glycine and were at the optimized pH (Table III). The thermograms of the IgMs in the optimized buffers are shown in Fig. 1ii.

Stability of the IgMs Under Stress Conditions

The IgMs showed less than 1% fragments and aggregates formation after exposure to mixing or 5 freeze-thaw cycles in the optimized buffers (Fig. 3i).

Table III Optimized Storage Buffers Based on DSC Measurements Which were Used for Accelerated and Real Time Stability Studies

	pH	Sorbitol [%]	Glycine [mM]
mAb 5	5.5	20	1000
mAb 14	5.0	20	1000
mAb 63	5.0	20	1000
mAb 84	7.5	20	1000
mAb 85	5.5	20	1000
mAb 95	7.0	20	1000
mAb 432	5.5	20	1000
mAb 529	5.5	20	1000

For comparison, IgMs were also subjected to stress in phosphate buffered saline (PBS), pH 7.4. mAb 529 was

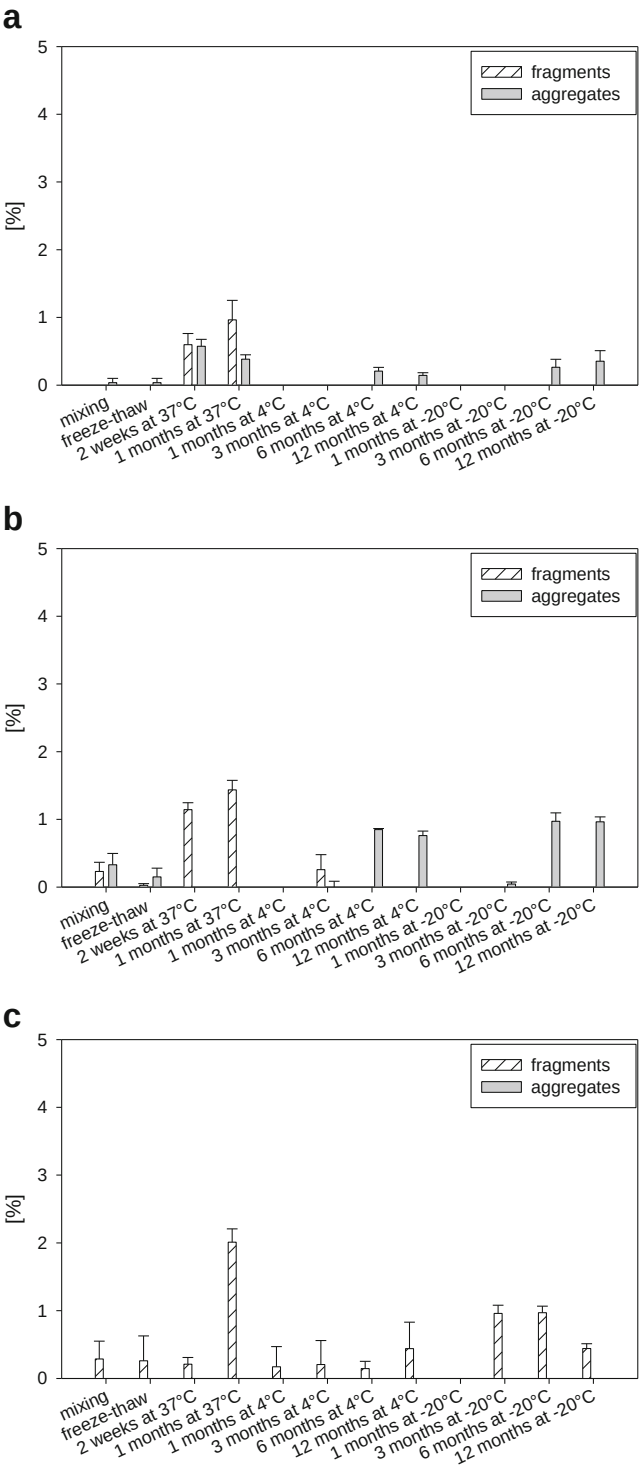

Fig. 3 Stability of the IgMs under stress, accelerated and real time storage in their optimized buffers. (**a**) mAb 5, (**b**) mAb 14, (**c**) mAb 63, (**d**) mAb 84 (*i*) at pH 7.5, (*ii*) at pH 5.5, (**e**) mAb 85, (**f**) mAb 95 (*i*) at pH 7.0, (*ii*) at pH 5.5, (**g**) mAb 432, (**h**) mAb 529. The fragment and aggregate formation was determined using SEC-UV.

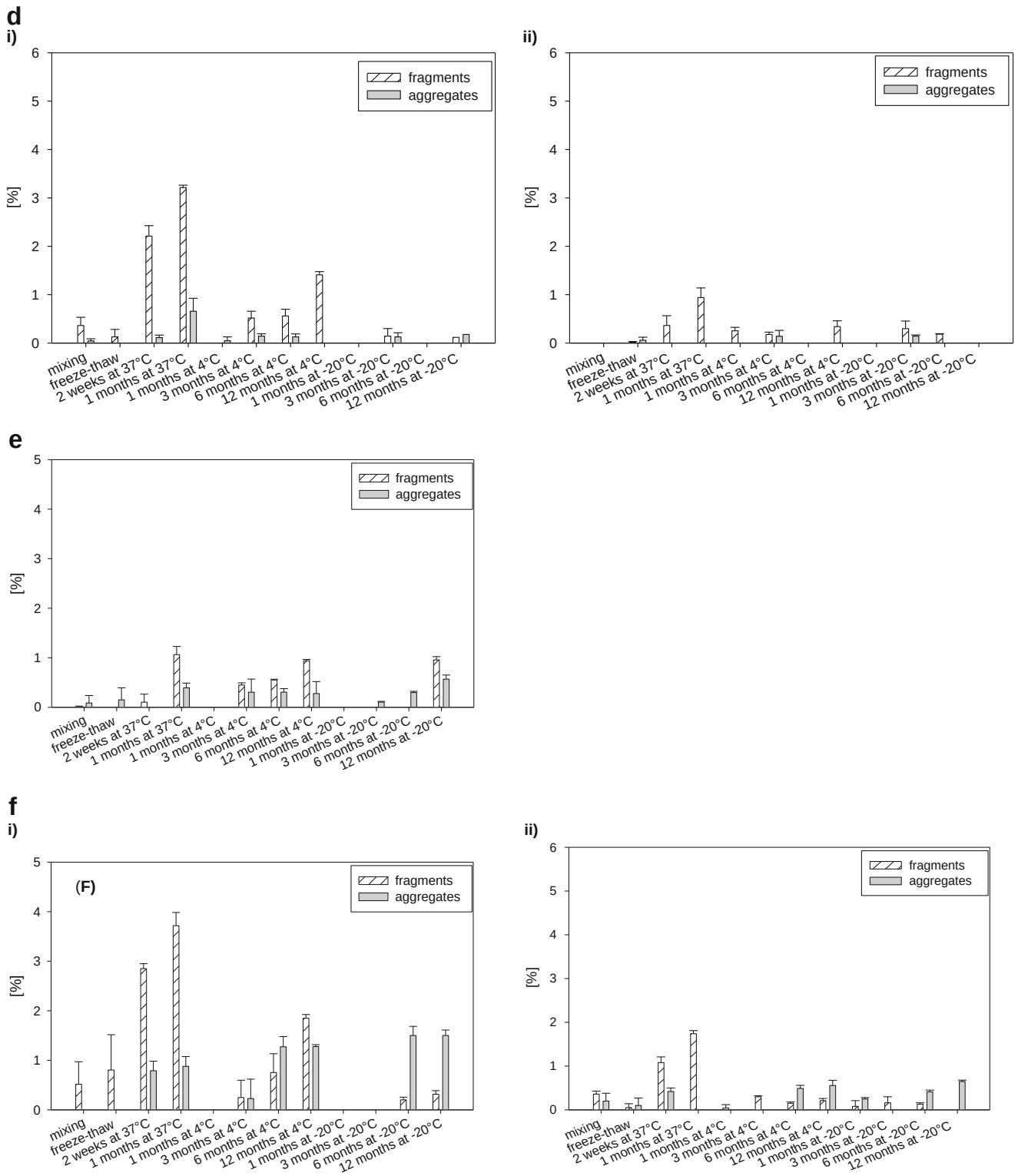

Fig. 3 (continued)

highly aggregated (34%) after 5 freeze-thaw cycles in PBS (data not shown). All other IgMs showed less than 1% fragment or aggregate formation in PBS during mixing and after 5 freeze-thaw cycles.

Stability of the IgMs Under Accelerated Storage

During accelerated storage at 37°C for 4 weeks, 3 out of 8 IgMs namely mAb 5, 85 and 529 formed less than 1% aggregates and

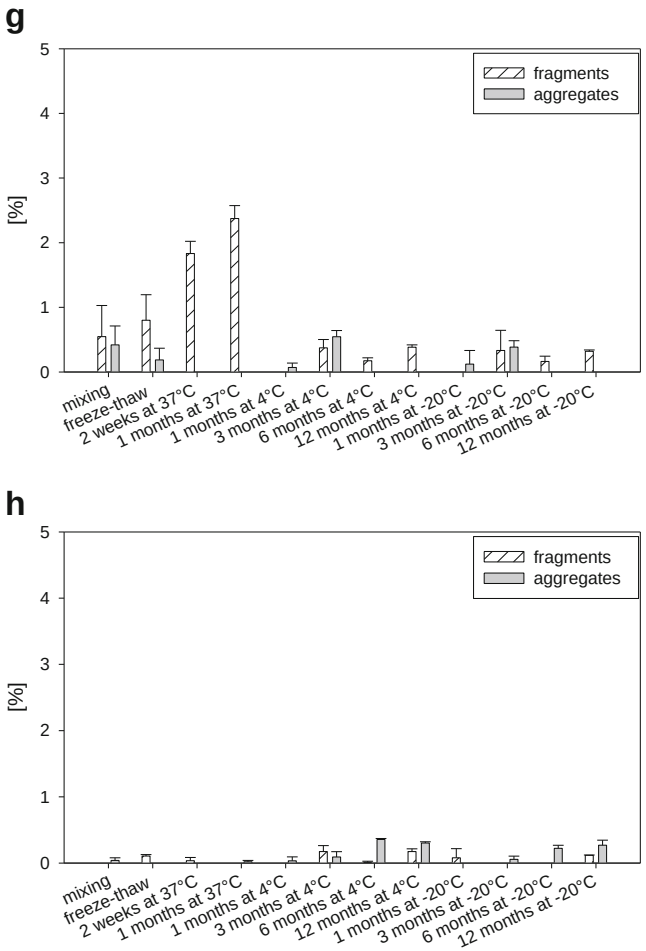

Fig. 3 (continued)

fragments in the optimized buffers (Fig. 3i). mAb 63 formed less than 1% fragments and aggregates for 2 weeks, but 3.3% fragments after 4 weeks. 4 out of 8 IgMs formed more than 1% fragments after 2 weeks: mAb 14 formed 1.6%, mAb 84 2.2%, mAb 95 4.4% and mAb 432 2.4% fragments (Fig. 3i) which increased slightly after 4 weeks: to 1.9% for mAb 14, 3.2% for mAb 84, 5.0% for mAb 95 and 2.9% for mAb 432, respectively.

mAb 84 and 95 were stored in buffers with higher pH than the other IgMs, (pH 7 and 7.5, respectively) and showed the highest degradation rate (Fig. 3i). So, the stability of these 2 IgMs in 20% sorbitol, 1 M glycine at pH 5.5 was tested. The fragment formation was reduced to less than 1% for mAb 84 and less than 2% fragments for mAb 95 after 4 weeks at 37°C (Fig. 3ii). This was compromising with conformational stability of mAb 95 and 84 at pH 5.5, which was 1.3°C or 2.5°C lower than at pH 7.0 and 7.5 (Table II).

Stability in the Optimized Buffer *Versus* PBS Under Accelerated Storage

The degradation rate of the selected examples mAb 84, 85 and 529 in the optimized buffer (20% sorbitol, 1 M glycine, pH 5.5) was significantly lower than in PBS (Fig. 4i, ii). After 6 months at 37°C, the remaining monomer content in PBS was 49% for mAb 84, 22% for mAb 85 and 56% for mAb 529. In optimized buffer, 83% monomeric mAb 84 was left, 96% monomeric mAb 85 and 96% monomeric mAb 529. The molecular weight of the degraded monomer decreased significantly more in PBS than in the optimized buffer as detected by SEC-SLS (Fig. 4iii) and native PAGE (Fig. 5). Interestingly, the molecular weight of the degraded monomer of mAb 85 in PBS seemed to increase again after 3 months storage. Those results were not in agreement with the results from native PAGE, which shows a decrease of the molecular mass (Fig. 5). We assume that we detect a partially unfolded mAb 85 in PBS using SEC-SLS.

Stability of the IgMs in Real-Time Stability Studies

The aggregate and fragment percentage of 6 out of the 8 IgMs remained less than 1% for up to 12 months storage at 4°C and −20°C at all time points (Fig. 3i). mAb 84 showed slight fragmentation after 12 months at 4°C (1.4% respectively). mAb 95 formed more than 1% aggregates at 4°C and −20°C already after 6 months. After 12 months, mAb 95 showed 1.3% aggregate and 1.8% fragments at 4°C and 1.5% aggregates at −20°C. After reducing the pH of the storage buffer to 5.5, mAb 84 and mAb 95 also contained less than 1% aggregates and fragments for all time points up to 12 months (Fig. 3ii).

Immunoreactivity After Long-Term Storage

The immunoreactivity of the IgMs to hESC was retained after 12 months storage at 4 and −20°C. There was no significant change in the binding of mAb 5, 14, 63, 85, 95, 432 and 529 indicated by a constant shift (Fig. 6). TRA_1-60 was always used as a positive control and always showed a comparable shift. The cytotoxicity of mAb 84 remained the same (Fig. 6). The amount of remaining viable cells did not change significantly between treatment with mAb 84 in the beginning of the storage and after 12 months storage (4.0–4.5% remaining cells *vs.* 3.2–4.8% remaining cells).

Small-Angle X-ray Scattering (SAXS)

Figure 7 shows the SAXS measurements for mAb 529 in two different buffers: 50 mM HEPES, pH 7.5 and optimized buffer (Table III). The blue line shows the scattering profile for mAb 529 in HEPES buffer while the red line shows the profile in the buffer with stabilizing compounds. The hypothetical scattering curve (2RCJ) (39) is closer to

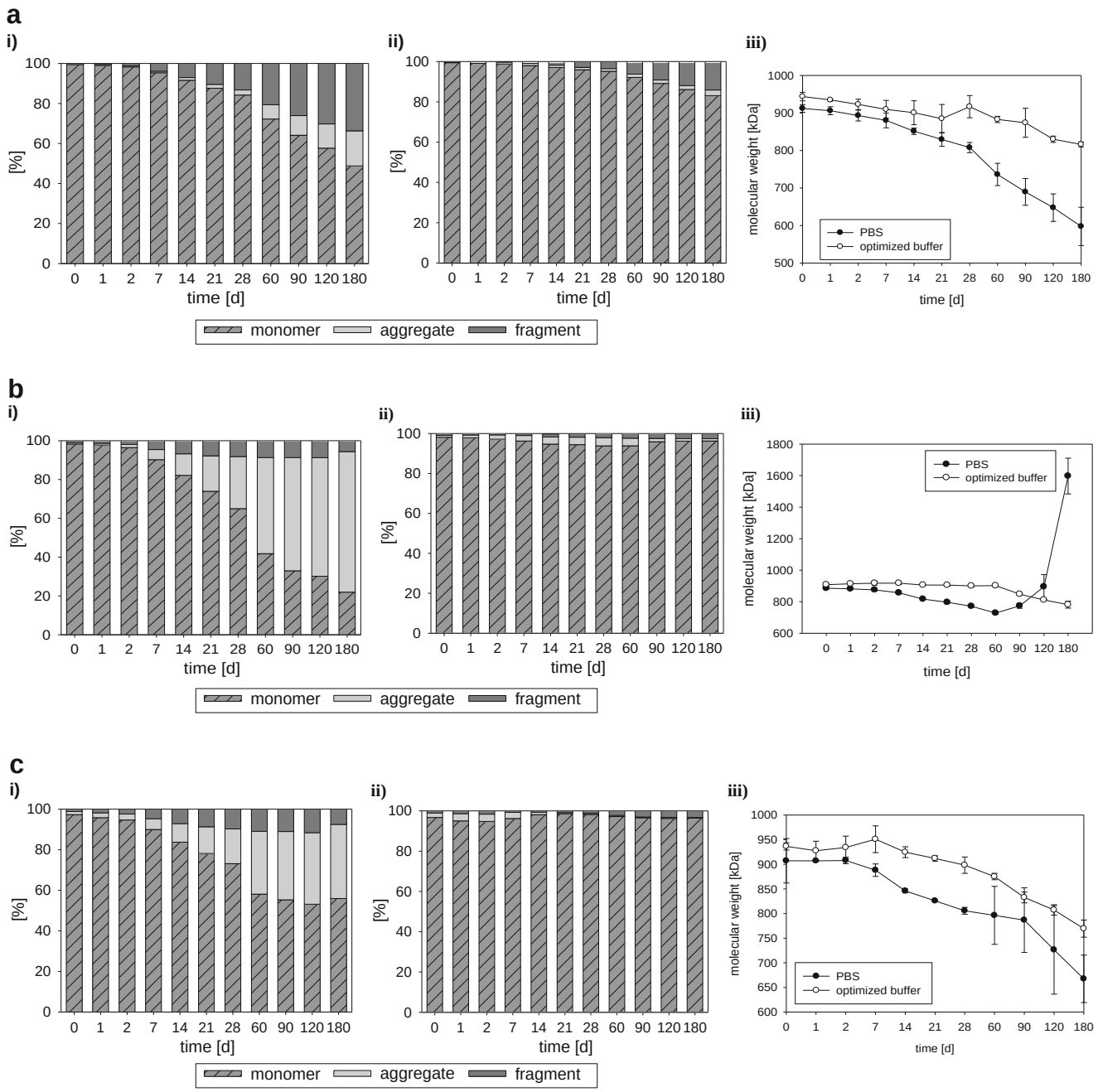

Fig. 4 Comparison of the stability at 37°C in (*i*) PBS and (*ii*) 20% sorbitol, 1 M glycine, pH 5.5: (**a**) mAb 84, (**b**) mAb 85, (**c**) mAb 529. *i*) and *ii*) show the percentage of aggregate and fragment formation analyzed by SEC-SLS, *iii*) shows the change of the molecular mass of the monomer over time, analyzed using SEC-SLS.

mAb 529 in the optimized buffer. Relative differences are used to calculate the changes in the conformation of antibodies in respect to the 2 different buffers. The reconstructed structural model shows minor deviations to 2RCJ. The change in respect to HEPES can be explained by a smaller opening angle (25°) see Fig. 7 insert. Thus it may be argued, that the optimized buffer stabilizes an IgM conformation similar to the one found for 2RCJ, while its pendant causes a small decrease of the opening angle.

DISCUSSION

A crucial challenge during manufacturing and storage of proteins is their long-term stability or shelf-life. Real time storage studies for developing stabilizing storage formulations are time consuming and costly. In this study, we used DSC combined with stress and accelerated storage studies to develop formulations suitable for long term storage of mouse monoclonal IgMs. Finally, we performed real time

stability studies for confirmation of the effectiveness of the formulations.

Using DSC in the first step, we obtained thermograms with one to three transitions. This finding correlated to literature reports of DSC data for IgMs (22). Due to a lack of data for IgMs, we conclude from studies with IgGs that the different transitions are caused by the different domains (Fab and Fc; or Fab, CH2 and CH3) (24).

The temperature of the first transition at pH 7.0 ranged between 60.4°C and 67.8°C. The values were lower than those reported in literature for human IgMs in PBS which ranged between 66°C and 72°C (6,22). The values were in the same range or higher than the T_m1 reported for mouse IgGs: 61°C (23) or 62°C (40).

For 3 out of the 7 IgMs (mAb 85, 432 and 529), the Tm was only slightly influenced by pH between pH 5.5 and 8.0. For the other 4 IgMs, the pH optimum for thermal stability was at or near the pI which ranged from ~5.5 to ~7.7 (Table I). It was shown previously that the maximal conformational stability was achieved at a pH near the pI if the pH dependent conformational stabilization is related to electrostatic effects (26,41). Attractive charge-charge interactions on the protein surface lead to protein stabilization and repulsive charge-charge interactions lead to protein destabilization (34).

Sorbitol, sucrose and glycine significantly increased the thermal stability of the IgMs. Sorbitol had a significantly higher stabilizing effect than sucrose, so only sorbitol was used in the optimized formulations. The T_m1 was increased up to 4–9°C by adding 1 M glycine and 20% sorbitol. Our results were in agreement with previously published studies regarding the stabilizing effect of sorbitol, sucrose and glycine (12,42). Indeed, a comparable increase of Tm of keratinocyte growth factor and IgG1 by sucrose and sorbitol was shown previously (12,42). Glycine exerted an even higher increase for keratinocyte growth factor (12), but no stabilizing effect on IgG1 (42).

The stabilizing effect by sorbitol and glycine increases linearly. The optimal excipient concentration was at the maximum concentration which was tested. Thus, the T_m further increases in the presence of even higher excipient concentrations (data not shown). Nevertheless the concentration was kept at 20% sorbitol and 1 M glycine because the viscosity becomes too high with higher excipients concentrations.

In this study, arginine decreased the thermal stability of all IgMs. For arginine preferential binding and preferential exclusion from a protein surface have been shown depending on the protein and on the arginine concentration (43). As mentioned previously, stabilizing cosolvents are preferentially excluded from the surface of a protein, thus leading to preferential hydradation and stabilization of a protein. In contrast, cosolvents that preferentially bind to proteins lead to a higher cosolvent concentration at the protein surface than in the bulk phase and thus destabilization of proteins. In this study, arginine seems to destabilize the IgMs *via* preferential binding.

Tween 80 did not significant increase the T_m and IgMs were stable under mixing and freeze-thawing without a surfactant. So Tween was not added to the final formulation since it can have adverse effects during storage. It may form peroxides which may lead to oxidation of the proteins (21).

In the next step, the storage stability in the optimized buffers was tested. It was found out that DSC was predictive for finding a stabilizing formulation for physical stress and storage for 6 out of the 8 IgMs. The optimized formulations sufficiently stabilized the

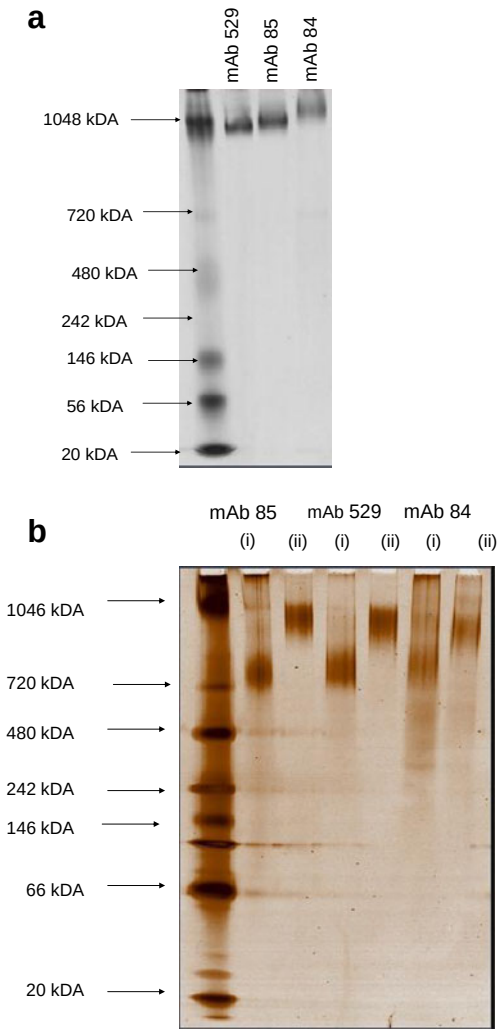

Fig. 5 Native PAGE of IgMs: (**a**) reference samples, (**b**) after 6 months storage at 37°C in (*i*) PBS or (*ii*) optimized buffer.

protein during shear stress, up to five freeze-thaw cycles, storage at elevated temperature and real time storage at 4°C and −20°C for up to 12 months.

The pH optimum for thermal stability of 2 IgMs (mAb 84 and 95) did not correspond to the pH optimum for preventing fragmentation and aggregation under accelerated storage and long-term real time storage studies. Both IgMs had a significantly higher fragment content than the other IgMs after 1 month at 37°C in buffer at pH 7.0/7.5. Reduction of the storage pH to 5.5 led to a significant reduction of the fragmentation and also aggregation. mAb 84 formed around 1.4% fragments after 12 months of storage at 4°C in pH 7.5 buffer which was reduced to less than 0.5% in pH 5.5 buffer. mAb 95 formed more than 3% fragments and aggregates in pH 7 buffer after 12 months storage at 4°C and 1.5% aggregates at −20°C. Reduction of the pH to 5.5 reduced the aggregation and fragmentation after 12 months storage at 4°C or −20°C to less than 1%. The higher aggregation tendency at pH 7.5 for mAb 84 and pH 7.0 for mAb 95 could be caused by the proximity to the pI. This observation is consistent with findings from previously published studies (26). Thus, in this study the pH optimum for conformational stability of mAb 84 and mAb 95 did not correlate to the optimum for low aggregation rate. As explained in the introduction, the pH maximum for high conformational stability and low aggregation rate do only correlate if the aggregation is related to protein unfolding (27). Our results are also in agreement with data from previously published studies regarding the higher fragmentation rate of antibodies at a pH between 7 and 8 after storage at elevated temperatures (11,44). A more recent review confirmed that the minimum of non-enzymatic fragmentation of antibodies is between pH 5.0 and 6.0 (45).

The accelerated storage studies at elevated temperatures were helpful and predictive to find a pH that was optimal for long term storage of mAb 84 and 95. The higher stability at lower pH (pH 5.5) for short term storage at 37°C correlated with higher stability at low pH at 4°C and −20°C for long term storage.

The storage stability in optimized buffers was also compared to the storage stability in PBS for 3 selected IgMs. The aggregation and fragmentation rate was much lower in optimized buffer than in PBS, even during accelerated

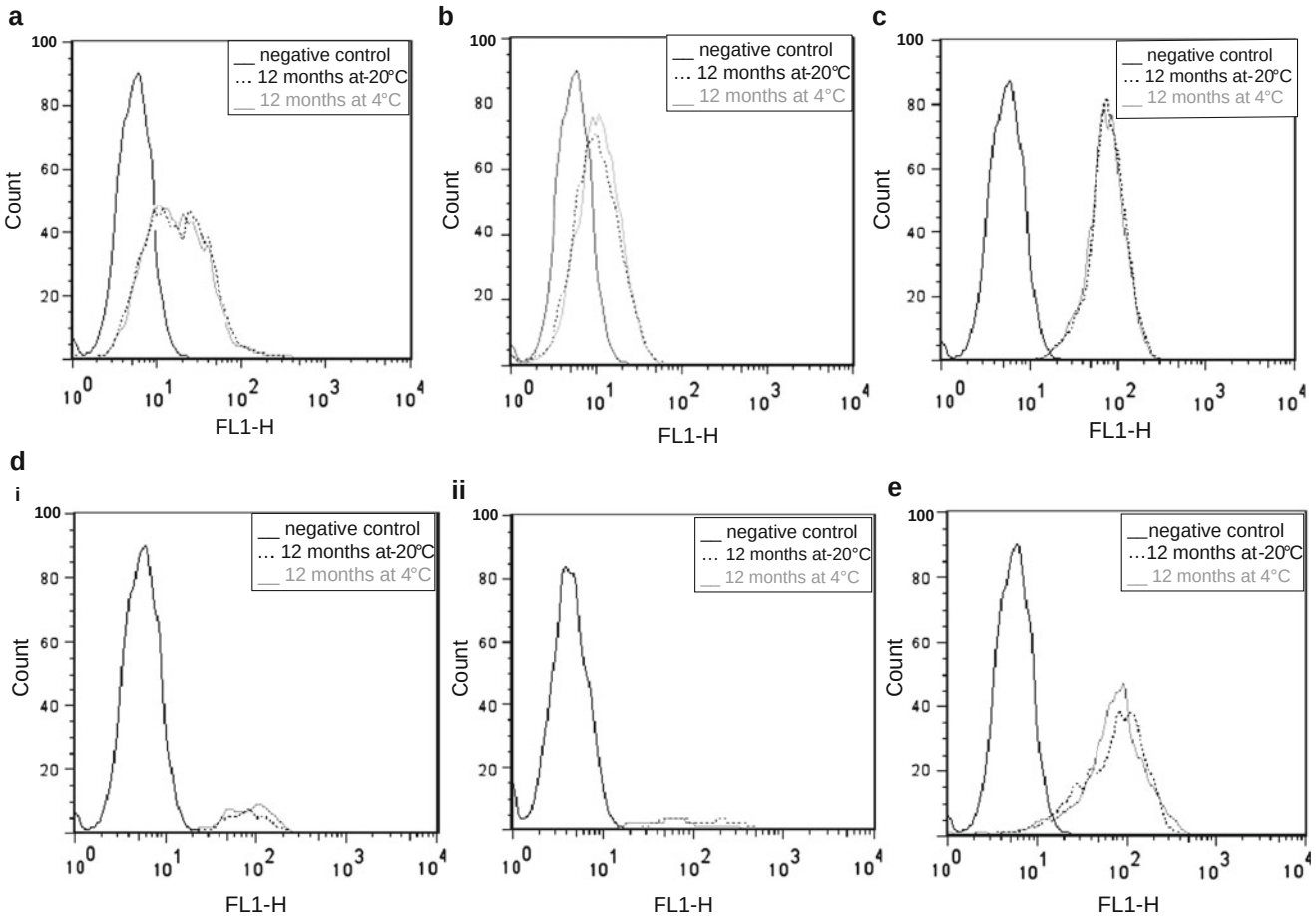

Fig. 6 FACS analysis of 12 months stored samples. (**a**) mAb 5, (**b**) mAb 14, (**c**) mAb 63, (**d**) mAb 84, *i*) pH 7.5, *ii*) pH 5.5, (**e**) mAb 85, (**f**) mAb 95, *i*) pH 7.0, *ii*) pH 5.5, (**g**) mAb 432, (**h**) mAb 529.

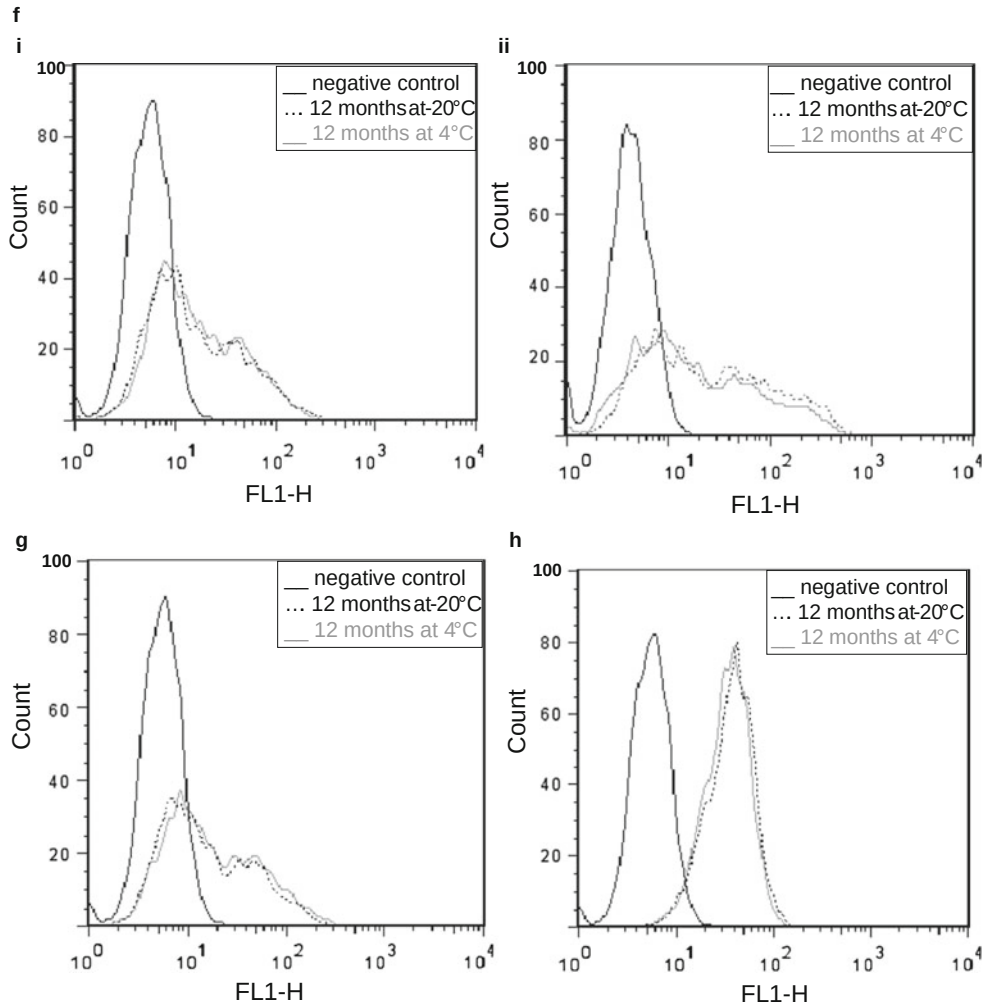

Fig. 6 (continued)

storage at 37°C for up to 6 months. Indeed, fragment and aggregate content of e.g. mAb 85 was 78% after 6 months at 37°C in PBS and only 4% in the optimized buffer. This study confirms that pH optimization and adding stabilizing excipients lead to significant reduction of aggregation and fragmentation during storage at elevated temperatures which is consistent with data from previous studies (11,12,45).

Small-angle X-ray scattering shows that the conformation of mAb 529 is similar to the hypothetical structure of IgM (2RCJ) by adding sorbitol and glycine thus leading to stabilization. The shift to higher scattering intensity of the IgM in HEPES buffer can be interpreted in two ways. Either an onset of forming aggregates or increased mobility of the domains could be responsible. This finding is in agreement with a previous study that showed a more compact native state with lower conformational mobility in the presence of excipients (46). This state may be less susceptible for degradation by chemical and proteolytic pathways.

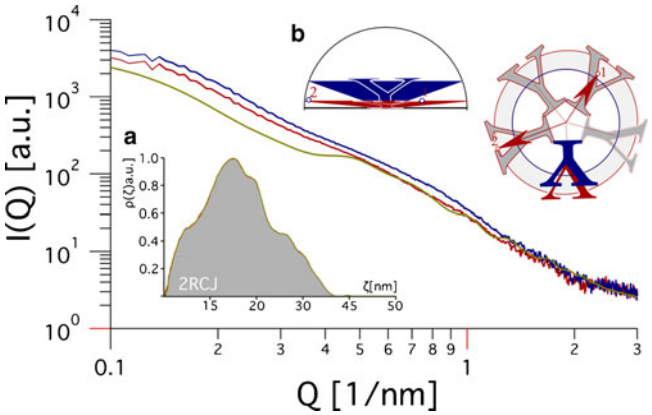

Fig. 7 Gives SAXS data for mAb 529 in HEPES buffer (*blue*) and optimized buffer (*red*). *Insert A* gives local density profile of the structure model of 2RCJ (*gray body*) while green line gives the local density profile on basis of which the green hypothetical scattering curve is reconstructed. Differences are minor. The deviation to the *blue line* is explained in terms of a more compact IgM structure model (see *insert B*). The IgM molecule does have a smaller opening angle.

CONCLUSIONS

A liquid formulation containing 20% sorbitol and 1 M glycine at pH 5.0–5.5 was found to stabilize 8 mouse monoclonal IgMs under shear stress, for up to 5 freeze-thaw cycles and under real time storage at −20°C or 4°C for 12 months. Those formulations may have broad utility for other IgMs. DSC was predictive for accelerated and long term storage for 6 out of the 8 IgMs studied. For 2 IgMs the pH optimum for conformational and storage stability did not correspond. Accelerated stability studies were helpful for finding the pH optimum for long term storage of these 2 IgMs. This study shows a concept for developing stabilizing formulations for IgMs which may be used for other proteins as well. SAXS measurements suggested a lower tendency to aggregate in the optimized buffer.

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