Journal of Immunological Methods 519 (2023) 113519

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Dried serum spots on pre-punched filter paper discs are ready-to-use storage and shipping devices for blood-borne antigens and antibodies

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ARTICLE INFO

Keywords:
Dried serum spots
Pre-punched filter paper discs
Submerge and dry protocol
Patient serum
Antibody analysis
Antigen analysis
Proteome analysis

ABSTRACT

Dried serum spots that are well prepared can be attractive alternatives to frozen serum samples for shelving specimens in a medical or research center's biobank and mailing freshly prepared serum to specialized laboratories. During the pre-analytical phase, complications can arise which are often challenging to identify or are entirely overlooked. These complications can lead to reproducibility issues, which can be avoided in serum protein analysis by implementing optimized storage and transfer procedures. With a method that ensures accurate loading of filter paper discs with donor or patient serum, a gap in dried serum spot preparation and subsequent serum analysis shall be filled. Pre-punched filter paper discs with a 3 mm diameter are loaded within seconds in a highly reproducible fashion (approximately 10% standard deviation) when fully submerged in 10 µl of serum, named the "Submerge and Dry" protocol. Such prepared dried serum spots can store several hundred micrograms of proteins and other serum components. Serum-borne antigens and antibodies are reproducibly released in 20 µl elution buffer in high yields (approximately 90%). Dried serum spot-stored and eluted antigens kept their epitopes and antibodies their antigen binding abilities as was assessed by SDS-PAGE, 2D gel electrophoresis-based proteomics, and Western blot analysis, suggesting pre-punched filter paper discs as handy solution for serological tests.

1. Introduction

Sampling blood, serum, or plasma from donors and patients is a medical routine. Accompanying blood marker analysis is well established, either by applying kits, which have been specially developed and proven safe by day-to-day handling experience, or through routine immuno-analytical procedures which have become popular in clinical laboratories (Benedicto et al., 2013; Grüner et al., 2015; Mair et al., 1992). Beyond medical routine testing, there is a need for screening proteins from serum or plasma as part of clinical studies. But here, storage and shipping conditions are of concern as the logistics of specimen handling in these circumstances are typically not standardized (Halvey et al., 2021; Newman et al., 2020). In many clinics in developed countries, preparation and freezing of serum and/or plasma have been established for storing and shipping clinical specimens to specialized laboratories (Pecks et al., 2012; Graham et al., 2017). This well practiced procedure requires initiating and keeping the cold chain directly after blood collection from the clinics or health care center to the laboratory (Shabihkhani et al., 2014). Since, for most investigations, pre-analytical handling is crucial, only a single thawing event at the laboratory is advised, ideally immediately before analysis (Huang et al., 2021). Multiple freeze/thaw cycles have been found deleterious for many quantitative determinations of markers and marker candidates, respectively (Halvey et al., 2021; Mitchell et al., 2005). Consequently,

Abbreviations: BSA, bovine serum albumin; DSS, dried serum spot; IRB, institutional review board; PVDF, polyvinylidene difluoride; SaD, Submerge and Dry; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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https://doi.org/10.1016/j.jim.2023.113519
Received 12 January 2023; Received in revised form 22 June 2023; Accepted 26 June 2023
Available online 6 July 2023

excess blood, serum, or plasma must be destroyed. Moreover, the constraints associated with the cold chain make it very difficult, if not impossible, to include less well-equipped physicians in private medical practices and/or clinics from rather remote places in international clinical studies or in studies where broader communities' involvement is required (Caldwell et al., 2018; Corran et al., 2008).

Dried blood spot (DBS) sampling has been developed to overcome the freeze and thaw-related bottleneck. It is applied in testing small molecules, such as metabolites and drug levels, as well as serum-borne proteins by employing various detection techniques, even in combination with mass spectrometry (Heiland et al., 2022; Li et al., 2020; Skjaervo et al., 2017). As an alternative, which is less often made use of, dried serum spot (DSS) or dried plasma spot (DPS) sampling has been reported to be well applicable for the investigation of intact serum proteins, *e.g.* with immuno-analytical methods (Caldwell et al., 2018; Maldonado-Rodriguez et al., 2017). The analysis of endogenous intact serum proteins by mass spectrometry eluted from DSS has recently been reported (Okai et al., 2021; Wölter et al., 2019). Moreover, proteins deposited on DSS were subjected to enzymatic digestion to generate peptide mixtures, which were then analyzed by mass spectrometry (Halvorsen et al., 2020). Each of these DSS/DPS-based analyses required the development of special procedures, considering both the solubility conditions required by the analytes, such as proteins, and the background tolerance limits imposed by the chosen analytical method.

Rather surprisingly, loading of analytes onto the DBS and/or DPS/DSS storage devices has not yet been standardized (Grüner et al., 2015; Caldwell et al., 2018; Tuaillon et al., 2020). Currently, blood is typically taken from adults and juveniles by venipuncture, finger pricks, or in the case of newborns from heel pricks. Several drops of blood are then collected on filter paper, with circled areas marking the deposition sites (Broberg et al., 2021; Jacobson et al., 2022). However, as loading is not controlled, deposited volumes can vary significantly. Equally problematic is serum deposition on filter paper by pipetting of unreported volumes. Then, pre-analytical ambiguity becomes unavoidable. Another concern is that in most cases, the materials for DBS and DPS/DSS preparations had been purchased from commercial sources, from which the respective specimen-loaded paper discs had to be cut out before re-solubilization of deposited analytes (Halvorsen et al., 2020; Whittaker et al., 2021). This cut-out or punching-out step increases the pre-analytical variability when the cut-out plug is not located in the center of the completely soaked DBS/DPS/DSS area, which - because of uncontrolled loading - may be different from the center of the marked circle. Because of this pre-analytical variability during sample loading and punching-out of the investigated filter paper discs, an alternative with accurately controlled DBS/DPS/DSS loading procedures is desired.

In this report, we describe the development and application of self-made filter paper discs which were pre-punched in a reproducible fashion to precise size for storage and shipping of defined serum protein quantities. Loading defined amounts of intact proteins and antibodies onto such pre-fabricated filter paper discs is guaranteed by fully submerging the filter paper discs in exactly pipetted volumes of serum (or plasma). After drying and storing the completely soaked filter paper discs at room temperature, the DSS prepared by this "Submerge and Dry (SaD)" protocol were stored for several days and then subjected to protein re-solubilization using optimized protocols to obtain accurately prepared protein solutions in each case. The re-dissolved serum-borne antigen proteins and antibodies were then tested for structural integrity and/or antigen-binding domains or ability by SDS-PAGE, 2D gel analyses, and Western blot analyses, respectively. The proteins used in this study cover a wide range of molecular masses, from apolipoprotein CIII (ApoCIII; approximately 9 kDa) and plasmodium falciparum merozoite surface protein 1_{19} fused onto maltose binding protein (MBP-pfMSP1_{19}; approximately 55 kDa) to antibodies (approximately 150 kDa). ApoCIII is a serum protein secreted by the liver and the small intestine and is found on triglyceride-rich lipoproteins, such as chylomicrons, very low-density lipoprotein (VLDL), and remnant cholesterol (Marshall et al., 2014). ApoCIII from maternal serum is of interest as a marker candidate of fetal growth restriction during pregnancy (Okai et al., 2020). The *pf*MSP1_{19} protein is found in human serum during acute malaria infection (Dijkman et al., 2021). The presence of anti-GPI (glucose-6-phosphate isomerase) autoantibodies in patient sera are markers of rheumatoid arthritis and other rheumatic diseases (Ronnelid et al., 2021). Establishing the SaD procedure with a standardized DSS method will facilitate wider use in clinical trials and future transition into clinical routine.

2. Materials and methods

2.1. Antibodies, antigens, and sera

The anti-*pf*MSP1_{19} antibody, the anti-ApoCIII antibody, the anti-mouse-IgG antibody from goat labeled with IRDye® 800 CW, the anti-rabbit-IgG antibody from goat labeled with IRDye® 800 CW, and the anti-human-IgG antibody from goat labeled with IRDye® 800 CW were obtained from commercial sources. The MBP-*pf*MSP1_{19} fusion protein was obtained from overexpression in *E. coli* (Opuni et al., 2020). The human GPI-GST fusion protein was a gift from the Immunology and Cell Biology Department of the University of Applied Sciences Bonn-Rhein-Sieg (Al-Majdoub et al., 2013). Bovine Serum Albumin (BSA) was purchased as a lyophilized powder. The rabbit serum was purchased from Kaninchenbetrieb Palleit (Warnkenhagen, Mecklenburg-Western Pomerania, Germany). Human donor serum (Wölter et al., 2016) and human patient serum (El-Kased et al., 2009) were obtained from the biobank of the Proteome Center Rostock. IRB numbers for the patient sera are EK138/06 (ApoCIII) and EV71/99 (anti-GPI), respectively. For reagent details, see Supplement.

2.2. The "Submerge And Dry (SaD)" protocol

Filter paper discs with 3 mm diameter were prepared by punching filter paper (580 × 580 mm, Gel-Blotting Paper, Schleier&Schuell, Keene, New Hampshire, USA) with a punch plier (Prym Group, Stolberg, Germany). For the loading of serum and/or protein solutions, one filter paper disc was placed inside a lid cavity of one 0.5 ml reaction tube (Eppendorf AG, Hamburg, Germany), which was kept upside-down. To generate DSS, the respective serum or protein solution (10 µl) was deposited on the filter paper disc, which had been placed in the reaction tube lid. The solvent was allowed to evaporate at room temperature for 4 h. Afterwards, the lid of the reaction tube was closed with the tube still turned upside down to ensure that the DSS remained in the tube's lid cavity at room temperature for 72 h (Okai et al., 2021). Alternatively, after DSS preparation, the filter paper disc was removed from the lid of a first Eppendorf tube lid with a pair of tweezers and placed inside a second 0.5 ml reaction tube's lid cavity to assess potential protein losses upon adsorption to the vessels' walls.

2.3. Re-solubilization of serum proteins from DSS

The Eppendorf tube with a DSS in its lid cavity was carefully opened, keeping it in the upside down position. Then, 20 µl of modified RIPA buffer (modified Radio-Immuno-Precipitation-Assay buffer: 50 mM Tris/HCl, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, and 150 mM NaCl; pH 7.4) (Ngoka, 2008) were added to the lid cavity to submerge the DSS. After 10 min of incubation time, the lid cavity of the reaction tube was covered with a 1 × 1 cm^2 mesh (Gaze, Holthaus Medical, Remscheid, Germany), which had been wetted with 20 µl modified RIPA buffer. Then, the tube was closed with the lid still facing downwards to ensure that the submerged filter paper disc and the buffer remained in the lid cavity, and the moistened mesh was fixed at its position on the lid above the solvent. The reaction tube, still upside down positioned, was gently shaken for 1 h using a Vortex Genie 2 shaker (Scientific Instruments, Bohemia, New York, USA). Afterwards,

the reaction tube was turned around with the closed lid facing upwards. For elution, the reaction tube was centrifuged at 10,000 rpm for 10 min using a Centrifuge Mini Spin® (Eppendorf SE, Hamburg, Germany). After collecting the eluate at the bottom of the reaction tube (volume approximately 17 to 18 µl), the filter paper disc alongside the mesh was discarded. The eluted protein solution was stored at 4 °C for one to five days (Okai et al., 2021).

2.4. Protein analyses

Protein concentrations of sera and DSS-eluted solutions were determined using the Bradford assay as described (Heitner et al., 2006). SDS-PAGE or 2D gel analysis was performed according to standard methods, and proteins were stained with Coomassie brilliant blue (Okai et al., 2021; Röwer et al., 2018; Laemmli, 1970; Kang et al., 2002; Konus et al., 2013; Postu et al., 2019; Kienbaum et al., 2009). Western blot analyses with DSS-eluted antigen or antibody proteins and antigen-containing sera or antibody-containing sera were performed using standard protocols (Okai et al., 2021; El-Kased et al., 2009; Röwer et al., 2018). The MBP-*pf*MSP1$_{19}$ protein concentration in rabbit serum was estimated to be 0.03 µg/µl. For details, see Supplement.

3. Results

3.1. General study concept and optimization of the "Submerge and Dry (SaD)" protocol

To develop our fast and highly reproducible "Submerge and Dry (SaD)" protocol, a test system was established which consists of model sera obtained from rabbits into which was spiked-in either a recombinant antigen or a monoclonal antibody, each in defined and controlled concentrations (Fig. 1A). Application examples included Western blot analysis of human ApoCIII as an endogenous antigen from human donor serum and anti-GPI autoantibodies in human patient serum (Fig. 1B).

Loading of serum onto pre-punched filter paper discs was done by entirely submerging the discs in serum which guaranteed saturation of the discs within a few seconds (SaD protocol). Since the discs with 3 mm and larger diameters did not reach the bottom of a 0.5 ml reaction tube (Eppendorf tube) and hence would have needed larger volumes of up to 100 µl for submerging, soaking of filter paper discs with serum was done in the lids of the 0.5 ml reaction tubes (Supplemental Fig. 1).

Because the lid cavities have flat bottoms, serum volumes of 10 µl were sufficient to completely submerge the 3 mm filter paper discs. Note, while filter paper discs with 2 mm diameters did not absorb the entire 10 µl volumes of serum, the 3 mm filter paper discs did. Once completely soddened, drying times of filter paper discs were determined by weighing, which showed that at room temperature, DSS from 3 mm filter paper discs were obtained within approximately 120 min (Supplemental Fig. 2 and Supplemental Table 1).

The modified RIPA buffer was chosen to elute serum proteins, including antigens and antibodies from DSS, since this buffer is compatible with immuno-analytical assays. Protein amount recoveries were around 90% (± 10%), as was determined by protein concentration determinations (Table 1, Supplemental Tables 2 and 3).

To test whether the tube lid walls had adsorbed substantial amounts of proteins in addition to the absorption of proteins by the filter paper discs, DSS preparation experiments were repeated, but the dried filter paper discs were transferred to new Eppendorf tube lids prior to eluting the proteins from DSS. Protein recovery after DSS transfer was comparably high, showing that most of the proteins had been absorbed by the filter paper disc after submerging them in 10 µl of serum. Adsorption to the tubes' lids' walls was negligible (Table 1, Supplement Table 4). The protein composition of human donor serum and that of the eluted solution after DSS preparation was estimated by 2D-PAGE analysis using a pH gradient between pH 4 and pH 7 and a mass range from approximately 220 kDa to 8 kDa (Supplemental Fig. 3). In the solutions eluted from DSS, the typical serum protein spots are found at the expected locations in the gels. Differences in spot intensities between two gels are within the methods' reproducibility margins.

3.2. Model studies with spiked-in antigen or antibody

The recombinantly produced MBP-*pf*MSP1$_{19}$ fusion protein was spiked-in into rabbit serum to mimic the presence of an infection-derived antigen in an individual's blood. Such prepared model serum either was applied as is to function as a positive control or was subjected to DSS preparation using the SaD protocol. Upon elution of proteins from DSS, a portion was loaded onto a 12% SDS gel (Fig. 2A) together with the positive control serum solution. The band pattern of serum proteins and that of eluted proteins from DSS turned out to appear nearly identical, with the strongest bands belonging to serum albumin at an apparent molecular mass of approximately 55 kDa. This result agrees with protein concentration determinations, indicating high protein yields after re-solubilization of DSS-deposited and stored serum proteins at room temperature.

Western blot analysis with monoclonal anti-*pf*MSP1$_{19}$ antibody as primary antibody and polyclonal anti-mouse IgG antibody from goat labeled with IRDye® 800 CW as secondary antibody generated indistinguishable band patterns for both protein solutions obtained after re-solubilization of proteins from the SaD-loaded DSS and fresh serum with spiked-in antigen (Fig. 2B). The antibody-decorated bands of the MBP-*pf*MSP1$_{19}$ fusion protein were found at an apparent molecular mass of approximately 45 kDa. Of note, the locations of the MBP-*pf*MSP1$_{19}$ fusion proteins in the gels are lower than expected. Based on our

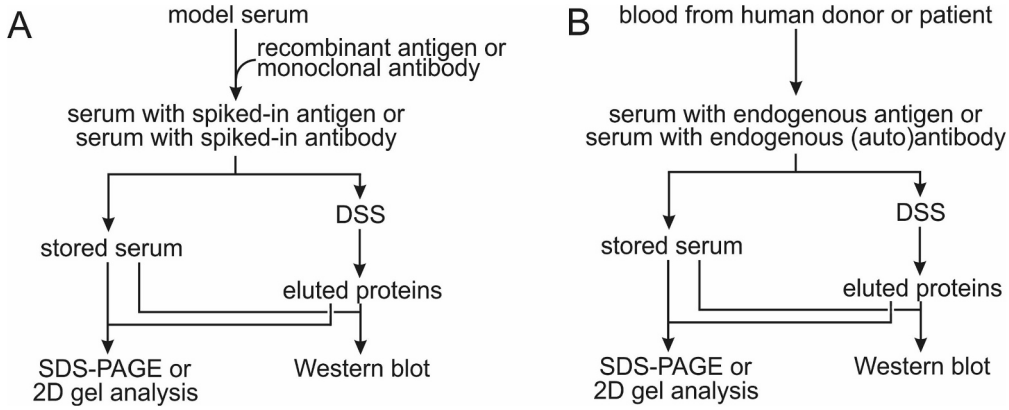

Fig. 1. Schemes of the "Submerge And Dry (SaD)" method development and application of the DSS preparation suitable for subsequent immuno-analytical methods. (A) Model serum with spiked-in antigen or antibody. (B) Human donor serum with endogenous antigen or human patient serum with endogenous (auto)antibodies.

Table 1
Preparation of DSS from Serum and Protein Elution from DSS.

Tube	Serum	Preparation of DSS				DSS	Protein elution from DSS				Protein
		Vol.[a]	Protein conc.[a]	Protein amount[a]	Dry time	Time period at RT[b]	Buffer vol.[a]	Recovered buffer vol.[a]	Protein conc.[a]	Protein amount[a]	Recovery[a]
(no.)		(µl)	(µg/µl)	(µg)	(h)	(h)	(µl)	(µl)	(µg/µl)	(µg)	(%)
9[c]	Rabbit[e]	10	48.6	486.0	4	72	20	15.4 ± 1.8	28.9 ± 2.2	444.9 ± 49.5	91.5 ± 10.2
5[c]	Donor[f]	10	56.7 ± 4.8	567.0 ± 47.5	4	72	20	18.8 ± 0.5	21.8 ± 3.1	408.8 ± 35.9	72.7 ± 10.2
5[c]	Patient[f]	10	56.7 ± 4.8	567.0 ± 47.5	4	72	20	18.6 ± 0.5	22.6 ± 2.7	419.3 ± 58.9	74.0 ± 9.1
2[d]	Rabbit[g]	10	44.4	444.0	4	72	20	15.5 ± 0.5	26.5 ± 0.4	410.2 ± 12.7	92.4 ± 2.8
8[d]	Donor[h]	10	68.7 ± 4.0	687.0 ± 39.6	4	72	20	17.5 ± 0.5	37.2 ± 2.0	650. 6 ± 25.2	94.8 ± 6.4

[a] Per DSS.
[b] RT: room temperature.
[c] DSS not transferred prior to elution.
[d] DSS transferred to new tube lid prior to elution
[e] Serum aliquots from one animal: for individual values see Table S2.
[f] Serum aliquots from several individuals; for individual values see Table S3.
[g] Serum aliquots from one animal: for individual values see Table S4.
[h] Serum aliquots from several individuals; for individual values see Table S4.

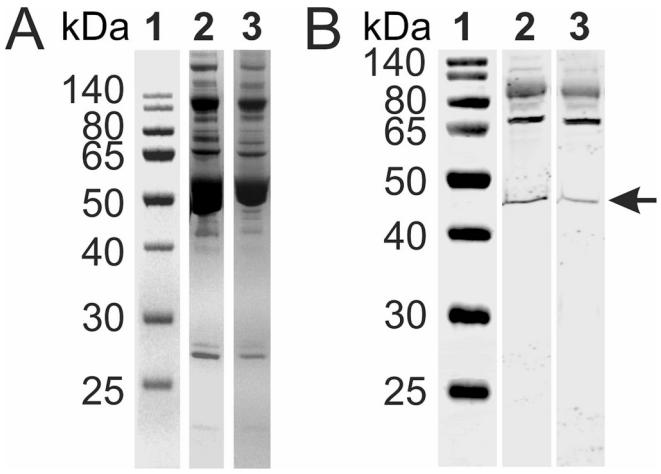

Fig. 2. Analysis of model serum with spiked-in antigen and from DSS eluted protein solution. **A:** SDS PAGE analysis of rabbit serum proteins with spiked-in recombinant MBP-*pf*MSP1$_{19}$ antigen. *Lane 1:* Molecular mass marker (PageRuler Prestained Protein Ladder). Apparent molecular masses are shown at the left. *Lane 2:* Stored serum from rabbit with spiked-in recombinant MBP-*pf*MSP1$_{19}$ fusion protein antigen. *Lane 3:* From DSS eluted protein mix after depositing rabbit serum with spiked-in recombinant MBP-*pf*MSP1$_{19}$ fusion protein antigen on filter paper disks. Proteins were stained with colloidal Coomassie Brilliant Blue. **B:** Western blot analysis of recombinant MBP-*pf*MSP1$_{19}$ antigen. *Lane 1:* Molecular mass marker (PageRuler Prestained Protein Ladder). Apparent molecular masses are shown at the left. *Lane 2:* Stored serum from rabbit with spiked-in recombinant MBP-*pf*MSP1$_{19}$ fusion protein antigen. *Lane 3:* From DSS eluted protein mix after SaD-loading of rabbit serum with spiked-in recombinant MBP-*pf*MSP1$_{19}$ fusion protein antigen on pre-punched filter paper disks. Monoclonal anti-*pf*MSP1$_{19}$ antibody was used as the primary antibody. Polyclonal anti-mouse IgG antibody from goat labeled with IRDye® 800 CW was used as a secondary antibody. The arrow on the right points to the location of decorated antigens. Background staining affords bands with molecular masses above 70 kDa. Blot images have been cropped above 140 kDa and below 20 kDa, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

extensive experience, we speculate that the large amounts of serum albumin in the protein solutions displaced the MBP-*pf*MSP1$_{19}$ fusion proteins, which then migrated to rather unusual positions. The additionally stained band at an apparent molecular mass of 75 kDa was assigned to represent MBP-*pf*MSP1$_{19}$ dimers. Of note, Western Blot analysis of the purified fusion protein alone also provided two bands, one around 80 kDa and one at approximately 55 kDa and protein identification results from tryptic in-gel digestion and mass spectrometric peptide mixture analysis confirmed presence of MBP-*pf*MSP1$_{19}$ therein (data not shown). The additional band at around 100 kDa indicates cross-reactivity of the secondary antibody to serum IgG, which was supported by further experiments analyzing rabbit serum without spiked-in MBP-*pf*MSP1$_{19}$ antigen (data not shown).

We next tested whether antibodies as part of the serum could still recognize their respective antigens after the SaD protocol, DSS preparation, and elution. To do so, we applied basically the same test system as introduced, *i.e.* rabbit serum formed the matrix which now was spiked with the monoclonal anti-*pf*MSP1$_{19}$ antibody. This arrangement was chosen to mimic seroconversion, *i.e.* an altered immune status upon infection recovery. The SDS-PAGE band pattern visualized by Coomassie blue staining of separated proteins from rabbit serum spiked with the monoclonal anti-*pf*MSP1$_{19}$ antibody was nearly identical to the band pattern obtained from the protein solution prepared by re-solubilization of proteins from DSS (Fig. 3A).

Next, the recombinant MBP-*pf*MSP1$_{19}$ antigen was loaded onto a 12% SDS gel. After electrophoresis, MBP-*pf*MSP1$_{19}$ was blotted onto PVDF membranes. After blocking the membranes, serum which contained the spiked-in monoclonal anti-*pf*MSP1$_{19}$ antibody and the protein solution obtained by DSS elution, which also contained the spiked-in monoclonal anti-*pf*MSP1$_{19}$ antibody, were applied as primary antibody-containing solutions.

The presence of the primary antibody on the blot membrane was visualized by adding the polyclonal anti-mouse IgG antibody from goat, labeled with IRDye® 800 CW, which was used as the secondary antibody. The positive and strong staining of the protein band at approximately 55 kDa apparent molecular mass, the location of the antigen, indicates the presence of intact, *i.e.* epitope-recognizing, antibodies in the solution eluted from DSS (Fig. 3B). A weaker band at approximately 100 kDa apparent molecular mass was assigned to be a homo-dimer of the antigen.

Encouraged by these results, we tested our DSS preparation, *i.e.* the SaD loading protocol and DSS elution procedure, with real-world human sera.

3.3. Application examples with endogenous antigen or antibody

As the first application example, we tested our DSS preparation using the SaD protocol by analyzing the presence of ApoCIII in human serum.

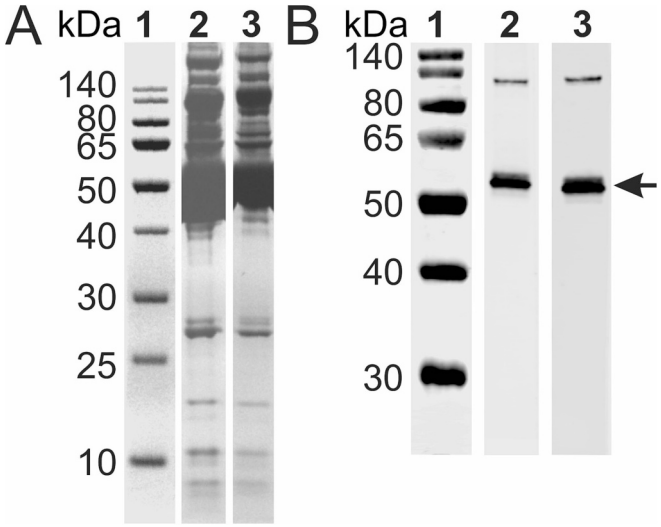

Fig. 3. Analysis of model serum with spiked-in antibody and from DSS eluted protein solution. **A:** SDS PAGE analysis of rabbit serum proteins with spiked-in monoclonal anti-*pf*MSP1$_{19}$ antibody. *Lane 1:* Molecular mass marker (PageRuler Prestained Protein Ladder). Apparent masses are shown at the left. *Lane 2:* Stored serum. *Lane 3:* From DSS eluted protein mix after having deposited rabbit serum with spiked-in recombinant MBP-*pf*MSP1$_{19}$ fusion protein antigen on filter paper disks. Proteins were stained with colloidal Coomassie Brilliant blue. **B:** Western blot analysis of recombinant MBP-*pf*MSP1$_{19}$ antigen. *Lane 1:* Molecular mass marker (PageRuler Prestained Protein Ladder). Apparent masses are shown at the left. *Lanes 2 and 3:* Recombinant MBP-*pf*MSP1$_{19}$ fusion protein antigen. *Lane 2:* Stored serum from rabbit with spiked-in monoclonal anti-*pf*MSP1$_{19}$ antibody was used as the primary antibody. *Lane 3:* From DSS eluted protein mix after "SaD"-loading of rabbit serum with the spiked-in monoclonal anti-*pf*MSP1$_{19}$ antibody on pre-punched filter paper disks was used as primary antibody. Polyclonal anti-mouse IgG antibody from goat labeled with IRDye® 800 CW was used as the secondary antibody. The arrow on the right points to the location of decorated antigens. A high molecular mass band shows a decorated antigen dimer. Blot images have been cropped above 140 kDa and below 25 kDa, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

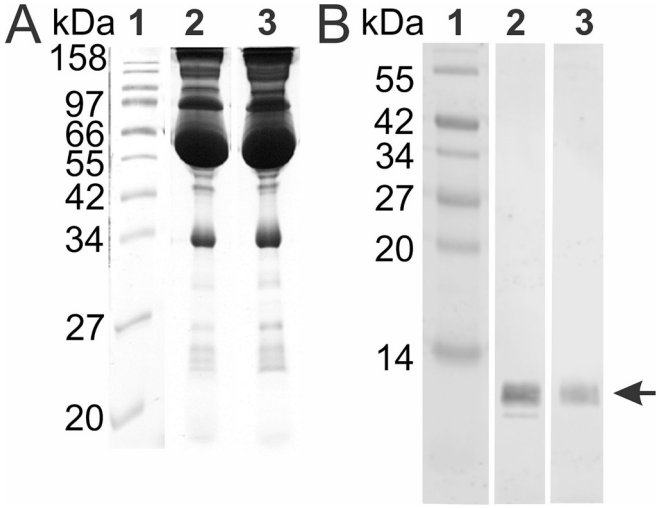

Fig. 4. Analysis of donor serum and from DSS eluted protein solution. **A:** SDS PAGE analysis of human donor serum proteins. *Lane 1:* Molecular mass marker (Broad range protein marker). Apparent masses are shown at the left. *Lane 2:* Stored human donor serum. *Lane 3:* From DSS eluted protein mix after having deposited human donor serum on filter paper disks. Proteins were stained with colloidal Coomassie Brilliant blue. **B:** Western blot analysis of human Apo CIII antigen. *Lane 1:* Molecular mass marker (Broad range protein marker). Apparent masses are shown at the left. *Lane 2:* Stored serum from human donor with endogenous ApoCIII protein as antigen. *Lane 3:* From DSS eluted protein mix after having deposited human serum on filter paper disks following the SaD protocol. Monoclonal anti-ApoCIII IgG antibody from rabbit was used as primary antibody. Polyclonal anti-rabbit IgG antibody from goat labeled with IRDye® 800 CW was used as secondary antibody. The arrow on the right points to the location of decorated antigens. Blot images have been cropped above 60 kDa and below 5 kDa, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Human serum was conventionally prepared in the clinics by centrifugation of blood after clotting. Then, the serum was aspirated and stored frozen. Two 10 µl aliquots of thawed serum were used as positive controls for either SDS-PAGE analysis or Western blot. Further, other 10 µl aliquots were deposited on pre-punched filter paper discs following the SaD protocol. Proteins eluted from DSS were subjected to SDS-PAGE analysis (Fig. 4A) and showed band pattern after Coomassie staining comparable to those from human serum (positive control).

Single bands at approximately 11 kDa were found in Western blot analyses with the monoclonal anti-ApoCIII IgG antibody from rabbit as the primary antibody and polyclonal anti-rabbit IgG antibody from goat labeled with IRDye® 800 CW as the secondary antibody in either case, *i.e.* in separated proteins from donor serum and from DSS eluted protein solutions (Fig. 4B). These results show that endogenous human serum antigens, such as ApoCIII, can be reliably detected in protein solutions obtained from SaD-loaded DSS.

To next answer the question of whether human autoantibodies from patient sera remained capable of binding their epitopes after deposition on filter paper discs using the SaD protocol, DSS preparation, and re-solubilization, we investigated a human patient serum containing autoantibodies directed against GPI. SDS-PAGE analysis and Coomassie staining of separated protein bands from either solution revealed nearly identical band patterns, showing that proteins derived from serum and proteins eluted from SaD-loaded DSS are fairly comparable (Fig. 5A).

As before, a Western blot assay was performed exposing the antigen (recombinant hGPI-GST fusion protein) either to patient serum (positive control) or to a protein mixture eluted from SaD-loaded DSS containing the anti-GPI autoantibodies. Decoration of the antigen by the primary antibodies was visualized by the addition of polyclonal anti-human IgG antibody from goat labeled with IRDye® 800 CW, which was used as a the secondary antibody (Fig. 5B).

In both cases, a single band was visualized that had migrated to an apparent molecular mass of approximately 70 kDa, which matches the expected location of the applied recombinant hGPI-GST fusion protein. No further bands were seen in the Western blots, demonstrating that the anti-hGPI autoantibodies present in human serum retained their specific binding capabilities after applying the SaD protocol for DSS preparation and following our elution procedure.

4. Discussion

Our aim was to test the potential of the SaD protocol for DSS preparation through qualitative experiments by demonstrating their applicability to serum proteins with different molecular masses. For research studies and future clinical applications, there is a need for quantitative reliability, best with automated analysis, which goes beyond this proof-of-principle study. Nevertheless, our study keeps the translational aspect, envisioning the challenges of clinical practice, such as, for example, prolonged storage times or variations of protein concentrations in patient sera, which were not investigated here. It must be kept in mind that the here reported antigen and antibody concentrations with the spike-in experiments are based on the assumed number of trophozoites in an acutely infected person (Perraut et al., 2005) and a molecular mass of 190 kDa for MSP1 (Some et al., 2018) together with educated guesses from long lasting experiences with serum protein

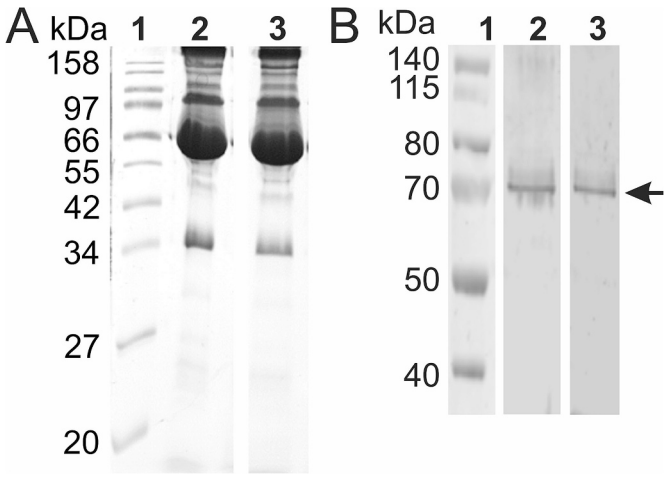

Fig. 5. Analysis of patient serum and from DSS eluted protein solution. **A:** SDS PAGE analysis of human patient serum proteins. *Lane 1:* Molecular mass marker (Broad range protein marker). Apparent masses are shown at the left. *Lane 2:* Stored serum. *Lane 3:* From DSS eluted protein mix after having deposited human patient serum on filter paper disks. Proteins were stained with colloidal Coomassie Brilliant blue. **B:** Western blot analysis of anti-human GPI autoantibodies. *Lane 1:* Molecular mass marker (PageRuler Prestained Protein Ladder). Apparent masses are shown at the left. *Lanes 2 and 3:* Recombinant hGPI-GST fusion protein antigen. *Lane 2:* Stored patient serum with endogenous anti-GPI autoantibodies was used as primary antibody. *Lane 3:* From DSS eluted protein mix after having deposited human patient serum with endogenous anti-GPI autoantibodies on filter paper disks was used as primary antibody. Polyclonal anti-human IgG antibody from goat labeled with IRDye® 800 CW was used as secondary antibody. The arrow on the right points to the location of decorated antigens. Blot images have been cropped above 140 kDa and below 40 kDa, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

analyses (Heitner et al., 2006; Koy et al., 2005; Pecks et al., 2010) and on experiments with mass spectrometry read-out after spiking in of antibodies (Zammataro et al., 2023). ApoCIII ELISA (Wölter et al., 2016; Wölter et al., 2012) as well as mass spectrometry data (Wölter et al., 2018) indicate that the ApoCIII concentration in plasma or serum is approximately 10 µM. The amount of spiked-in MBP-*pf*MSP1$_{19}$ fusion protein is in the same range (approximately 1 µM). Although the chosen concentration dimensions seem plausible, it cannot be ruled out that *in vivo* concentrations of antigens and antibodies, which may differ from patient to patient, ultimately might fall below the lower limits of detection.

For the preservation of peripheral blood without demanding to apply sophisticated equipment, the DBS technology has been implemented as a convenient tool for both qualitative and quantitative biological analysis (Malsagova et al., 2020). The extension of the DBS technology to DSS preparation is a logical next step and has been suggested to follow DBS-associated claims, as long as pipetting errors are avoided. Serum is obtained by simply keeping the collected blood in a container for approximately 60 min to ensure that clotting is complete. Then, the formed blood clot is centrifuged for about 10 min at 1000 *g* to 2000 *g* (Mercatali et al., 2018), and the serum presents as yellowish supernatant. The required centrifugal force is easily obtained by hand-powered centrifuges (Byagathvalli et al., 2019), allowing subsequent preparation of DSS by SaD-loading even in electricity-free regions. To simplify the method and improve accessibility further, finger prick blood collection may be considered instead of venipuncture. The blood volume obtained by a finger prick blood collection (Vasconcellos et al., 2020) is sufficient for the preparation of several DSS using the SaD protocol. Whereas the initial preparation of serum from blood can be done by any clinic or doctor's office, proper handling of neat serum can be difficult either due to lack of knowledge or resources. To avoid irreproducible experimental results, treatment of neat sera requests to precisely follow standard operating procedures (SOPs) (Tuck et al., 2009). However, implementing "good clinical practice (GCP)" and/or "good laboratory practice (GLP)" routines are a challenge for many healthcare facilities around the world, which starts with sample handling, in particular when freezing of specimen is required, but extends to the absence of qualified staff, sufficient funding, or even expands to political instability and insecurity (Ravinetto et al., 2016). To enable healthcare centers and physicians in private practice to contribute to clinical and pharmaceutical research, specimen collection and donor/patient documentation requests must be kept easiest, with very little demands on (clinical) staff, while still guaranteeing acceptable specimen quality. Our SaD loading of pre-punched filter paper discs fulfills all these demands. With our procedure of soaking pre-punched filter paper discs in adequate serum reservoirs and defined serum volumes for generating DSS (SaD protocol), the remaining crucial and error-prone loading step has now been investigated with this proof of concept study. Hence, our here-described and tested DSS preparation procedure should help to increase the acceptance to participate in internationally renowned clinical studies by physicians in private practice as well as by health care specialists, including those from remote health care institutions, for the benefit of their probands and patients.

5. Conclusion

Serum volumes and filter paper disc dimensions for DSS preparation by the SaD protocol are results of compromises between ease of handling, such as reproducible and error-minimized pipetting of serum, feasible soaking and drying times, and serum consumptions per patient/donor for subsequent immuno-analytical assays. Repetitions of preparations have been conducted with all three types of specimen examined in this study: animal model serum, human donor serum, and human patient serum (Table 1). While protein recoveries from human sera tended to be lower, at around 70% - 90%, compared to model sera, at around 90% - 95%, reproducibility was comparable with deviations of about 10% in each case. From our test series, the combination of minimal serum volume and ideal filter paper disc diameter was found with 10 µl of serum, which was sufficient to entirely submerge the pre-punched filter paper discs with 3 mm diameter, which leave no substantial superfluous residuals adsorbed to the vial walls after drying. Elution of proteins from DSS was done in the laboratory by adding 20 µl of modified RIPA buffer, resulting in protein solutions which could easily be handled by subsequent analytical procedures, such as SDS-PAGE, 2D gel-based proteome analyses, and Western blot analyses.

Funding

The DAAD is acknowledged for funding a stipend for C.A.O. (91614177).

Declaration of Competing Interest

None.

Acknowledgements

We thank H. Bäßler, A. Lemken, Dr. P. Lorenz, and Prof. H.-J. Thiesen from the Institute of Immunology at the University Medicine Rostock for their help in expressing the MBP-*pf*MSP1$_{19}$ fusion protein.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2023.113519.

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