Silver nanoparticles can be sampled by ultrafiltration probe but elution into & recovery from plasma and DPBS differs *in vitro*

Marije Risselada[1*], Robyn R. McCain[2], Miriam G Bates[1], Makensie L Anderson[1]

* Corresponding author

E-mail: mrissela@purdue.edu

[1]Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Purdue University, West Lafayette, Indiana, United States of America

[2]Center for Clinical Translational Research, College of Veterinary Medicine, Purdue University, West Lafayette, Indiana, United States of America

Abstract

We compared 1) the influence of elution fluid on rate, pattern, and completeness of silver nanoparticle (AgNP) elution, and 2) ultrafiltration (UF) probe and direct sampling *in vitro*. Six specimens (2.5ml of 0.02mg/ml 10nm AgNP and 5.0ml of 30% poloxamer 407) contained in a dialysis tube (12-14kDa pores) were placed in 100ml Dulbecco's Phosphate Buffered Saline (DPBS) (n=3) or canine plasma (n=3) for 96h on a stirred hot plate (37°C and 600rpm) and sampled 20 times. Six pipette and UF probe samples were taken of a 0.001mg AgNP/ml DPBS or plasma solution. Inductively coupled plasma mass spectrometry was used to analyze Ag. Stock plasma contained Ag. At 96h, 5/6 dialysis tubes had not fully released AgNP. One peak in hourly Ag increase was present in DPBS (10-13h), and two peaks in plasma (6-8h and 10-13h). The hourly Ag increase in plasma decreased earlier than in DPBS. UF probe sampling was possible in both DPBS and plasma and resulted in higher Ag concentrations but with more variation than pipette samples. While *in vitro* use of DPBS might be more cost effective, plasma should be considered due to difference in elution and recovery.

Introduction

With the increase in antimicrobial resistance in veterinary medicine [1,2], novel strategies and antimicrobials to combat infections are (re)gaining favor. One strategy might be to deliver a high local dose of antibiotics [3-7]. Another might be to explore the use of non-antibiotic antimicrobials, such as silver (Ag), especially silver nanoparticles (AgNP). They have received considerable interest for their use against micro-organisms [8,9] and in wound care [10]. Prior silver elution studies have been performed into phosphate-buffered saline (PBS) from poloxamer 407 [11], into deionized water baths (from silver oxide films) [12], or aqueous samples with varying NaCl concentrations (from solid coated specimens) [13]. Plasma might be a closer *in vitro* fluid approximation to an *in vivo* environment than PBS and could mimic protein interactions that might be encountered. The elution into human plasma from an antibiotic containing polymethyl methacrylate (PMMA) construct as well as the effect plasma on PMMA properties was reported [14]. However, plasma has not been evaluated for suitability of Ag release studies, and whether the benefit could outweigh the increased cost of commercial canine plasma (~$2,400/500ml; Innovative research, Novi, MI) over PBS (~$30/500ml; Gibco, Thermofisher Scientific, Billings, MT).

The ultimate goal of sustained release compounds assessed by elution studies is their *in vivo* application. Ultrafiltration (UF) probes have been used successfully in dogs [15,16] and are used to sample compounds present in the tissue, without the need for more invasive sampling methods, such as tissue biopsies [17]. Establishing the feasibility of sampling Ag with UF probes would be preferable prior to their use to investigate the *in vivo* pharmacokinetic profile of Ag containing local delivery products. However, the possibility of sampling AgNP via UF probe has

50 not been assessed, nor has a comparison of canine UF probes and direct sampling been reported
51 for AgNP.
52 We aimed to 1) determine and compare the influence of elution fluid on rate, pattern, and
53 completeness of AgNP elution *in vitro*, and 2) compare UF probe sampling with direct samples.
54 We hypothesized that 1) elution of AgNP into Dulbecco's PBS (DPBS) will be like elution in
55 canine plasma, and that 2) UF probe sampling will be constant over time and yield similar results
56 as direct sampling.

Materials and Methods

An observational elution study and repeat sampling study were performed separately. Canine Ultrafiltration Probes (BASi Instruments, West-Lafayette, IN) were assembled per manufacturer instructions [18].

Elution

Six elution specimens were prepared. Each contained 2.5ml of a 0.02mg/ml 10nm silver nanoparticles (AgNP) in aqueous buffer with sodium citrate as stabilizer (Sigma Aldrich, Saint Louis, MO) mixed with 5.0ml 30% poloxamer 407 (Pluronic® F-127, Sigma Aldrich) (1:2 ratio with a total Ag content of 0.05mg/specimen). The specimens were prepared in individual 12ml syringes <2 hours before use and stored refrigerated and shielded from light. Individual 150ml crystallization dishes (Synthware glass, Pleasant Prairie, WI) with 100ml fluid were prewarmed for 2 hours prior to starting the elution study. Three dishes had Dulbecco's phosphate-buffered saline without Calcium chloride or magnesium chloride added (DPBS, Gibco, Thermofisher Scientific, Billings, MT) and three had canine plasma with dipotassium EDTA (K2-EDTA) (IGCNPLAK2E500ml Canine Plasma lot 41273, Innovative research, Novi, MI). A 10 multi-position hotplate with magnetic stir bars was used (RT 10, IKA Magnetic Stirrers, Wilmington, NC) with settings at 37°C and 600 rpm throughout the experiment. Room temperature was set at 68°F. Specimens were created using a 10cm long strip of 1 inch dialysis tubing with 12-14kDa pores (Carolina Biological Supply Co, Burlington, NC) [19]). The distal free end was folded up length wise and secured in folded position using 2 large surgical stainless steel hemoclips (Hemoclips® Teleflex, Morrisville, NC) in opposite direction 11]. The Ag-poloxamer mix was then placed in the tube, and the proximal free end of the tube folded and closed in similar fashion. All specimens were created in one batch and then placed in the prewarmed DPBS or

canine plasma within 5 minutes after assembly. The t=0 sample was taken immediately after all specimens were submerged in the same order of assembly and placement. Twenty samples (0.15ml each) were taken over 96 hours with a decrease in frequency (0, 1, 2, 3, 4, 5, 6, 8, 10, 13, 17, 22, 27, 34, 42, 48, 58, 66, 72, 96 hours) with the sampling order consistent throughout. At 96 hours, an additional sample (by needle aspiration through the tube) of the fluid contained within the dialysis tube was taken.

Ultrafiltration probe sampling

Two solutions were prepared immediately prior to sampling in a 50ml conical centrifuge tube (Thermofisher Scientific) using graduated pipettes (Thermofisher Scientific) and a 3ml syringe (Covidien, Mansfield, MA). Each specimen contained 0.03mg of AgNP (1.5ml) total in 28.5ml of either DPBS or canine plasma for a fluid concentration of 0.001mg/ml of AgNP. Ultrafiltration probes were tested for patency and sampling using DPBS prior to use. Full submerging of the probe near the lowest point of the tube was ensured, and positioning was checked after each manipulation. Vacutainers without additives (Vacuette Blood collection tube, 3.0ml, no additive, Greiner Bio-One, Sigma Aldrich, St Louis, MO) were used to collect the probe samples and an additional 10ml of air was removed to increase negative pressure in each vacutainer to increase sampling speed. The initial sample of the specimen was discarded to avoid risk of dilution by plain DPBS. Repeat samples using both a UF probe and direct sampling were taken at 0, 5, 10, 15, 30 and 60 minutes.

Samples of stock AgNP, DPBS and plasma were taken prior to the start of the study. All samples were stored at -78°C until batch analysis.

Sample and data analysis

The quantity of Ag in each sample was determined via inductively coupled plasma mass spectrometry (ICP-MS) as previously described [11]. DPBS samples were diluted in 2% HNO3 and plasma samples were digested over night at 70C in the 1:1 mixture of 70% HNO3 and H2O2 and analyzed using ICP-MS (Perkin Elmer NexION 300D) to determine the concentration of silver within each sample. The short-term precision was less than 3% relative standard deviation (RSD), and the long-term stability was <4% relative standard deviation over 4 hours. Isotope-ratio precision was less than 0.08% relative standard deviation. The AgNP detection limit was 0.001ng/ml, and quantification limit at 0.002ng/ml, and all samples below this limit were recorded as 0ng/ml. Silver concentrations are expressed in parts per billion (ppb), with 1ppb=1ng/ml. Hourly increase in Ag was calculated as the difference between the measured values at two subsequent time points divided by the hours between time points (expressed as ppb/hr). The data of the 3 elution specimens will be expressed graphically as mean±SD. The values of the repeat sampling experiment will be reported as individual results and a mean±SD (RSD) in an observational manner without further statistical analysis. The RSD was calculated by dividing the SD by the mean and will be expressed as a %.

Results

Silver concentrations for stock solutions were: DPBS 0.19ppb; plasma 4.83ppb and AgNP 24,940ppb. Assembled UF probes initially did not reliably yield appropriate negative suction to obtain a sample, and probes were not re-usable for repeat experiments. Additional negative pressure applied to the vacutainers together with sealing connecting points with glue (3g Tube, The Gorilla Glue Company, Cincinnati, OH) allowed single use sampling.

Elution

No leakage of any dialysis tubes was observed under gentle pressure immediately after assembly. Five out of 6 dialysis tubes were fully filled at 96 hours, the sixth was not fully filled when retrieved. The five fully filled specimens still contained more Ag than the surrounding fluid (Table 1), and release of Ag was not complete at 96 hours. A burst release of Ag was seen both into DPBS and plasma in the first 13 (DPBS) and 8 (plasma) hours, with the baseline amount in plasma higher than in DPBS (Fig 1). The highest hourly increase in Ag was between 10-13 hours in both fluids, with a clear single peak in DPBS (20.66ppb/hr) whereas two peaks were present for plasma: between 6-8 hours (12.31ppb/hr) and between 10-13 hours (13.85ppb/hr). The hourly increase of Ag in DPBS tapered between 27-84 hours but continued throughout. The increase in Ag in plasma decreased earlier, between 8-60 hours, and measured Ag decreased thereafter. The amount of Ag measured at 96 hours in fluid and the specimens was higher in DPBS than in plasma (Fig 2).

Fig 1: A. Elution of Ag into either DPBS (light grey) or plasma (dark grey), expressed as ppb over 96 hours. The elution of Ag into DBPS continued, leading to a higher fluid concentration, whereas the increase of Ag in plasma tapered between 24 – 60 hours and Ag decreased after 60 hours. B. The increase in Ag is expressed as Ag (ppb)/hour measured over the timeframe prior to

the sampling time. A burst elution pattern is evident for both elution fluids. C. Elution of Ag into either DPBS or plasma, expressed as ppb over the first 22 hours. D. Hourly increase in Ag over the first 22 hours.

Fig 2: A. Ag amount (ppb) in elution fluid and remainder of the specimen at 96 hours. Measured Ag content in both the fluid and specimen remnant were less in plasma than DPBS. B. Pipette and ultrafiltration (UF) probe sampling of 1,247ppb Ag in DPBS and plasma solution. Both methods, in both mediums underestimated the Ag content. The UF probe samples had a wider variation in sampled Ag. The dotted line indicates 1,247ppb Ag.

Table 1 Silver (Ag) at 96 hours expressed as parts per billion (ppb).

Ag in DPBS		**Ag in Plasma**	
Fluid	**Specimen**	**Fluid**	**Specimen**
2137.97*	203.32*	25	32.34
293.18	512.65	20.02	68.29
248.75	528.26	23.36	58.90

Each tube contained 6660ppb Ag at the start of the experiment. *denotes the sample that was not fully filled and the surrounding fluid.

153 # Ultrafiltration probe sampling

154 Anticipated Ag content based on the stock (24,940ppb Ag (AgNP stock) diluted in 30ml) was
155 1,247ppb Ag of the diluted fluid. Ultrafiltration probes were able to collect Ag in both DPBS and
156 plasma, with Ag content in fluid obtained via UF probe sampling higher than the corresponding
157 pipette samples (Fig 2). However, the UF probe-obtained samples had more variation and values
158 differed between sampling times and decreased over time (Table 2). Samples obtained by both
159 methods had a measured Ag lower than anticipated, with the underestimation greater in DPBS
160 than plasma.

161 Table 2: Pipette and ultrafiltration (UF) probe sampling of a planned 1,247ppb Ag in DPBS and
162 plasma solution.

Repeat sampling of Ag (ppb)	**DPBS**		**Plasma**	
Time (min)	**UF probe**	**pipette**	**UF probe**	**pipette**
0	502.67	1.19	791.28	116.19
5	48.31	11.31	946.58	99.18
10	33.88	0.93	370.54	225.08
15	15.47	3.18	117.41	74.07
30	2.33	0.80	1111.30	116.02
60	3.95	6.93	785.83	89.27
mean±SD (RSD)	101.10±197.53 (195%)	4.06±4.25 (105%)	687.2±372.2 (54%)	119.9±53.9 (45%)

163 **Table 2**. Probes were allowed to sample for 4 minutes to obtain enough sample volume. Direct
164 samples were taken using a pipettor at the time point. UF probe samples were taken by attaching
165 a vacutainer to the probe for 4 minutes at the start time. DPBS= Dulbecco's Phosphate Buffered
166 Saline, UF= ultrafiltration. The mean is provided with both standard deviation (SD) and relative
167 standard deviation (RSD).
168

Discussion

Elution into DPBS yielded different results than elution into plasma, with a longer sustained initial hourly increase in fluid Ag and more variation in Ag measurements with a resultant less smooth curve. A burst release pattern was evident for both DBPS and plasma, which corresponds with an earlier study where ~88% of the Ag released from poloxamer 407 within the first 24 hours [11]. While the pore size of the dialysis tube should allow AgNPs to cross freely, the Ag concentration within the dialysis tube was still higher at 96 hours than the surrounding fluid, indicating incomplete release of the specimen in both elution media. Silver could be measured in DPBS and plasma by UF probe sampling.

The higher initial values of Ag found in plasma might be explained by Ag measured in stock plasma vs DPBS solution. It was then followed by an increase of Ag content similar in shape to the DPBS curve. However, this initial starting value would mask the initial release of Ag at the time when release will be highest and is a limitation of using plasma as the elution medium. We chose to report the measured values as-is instead of detracting the value obtained from stock plasma, as we only had a single measurement for plasma rather than repeat samples and felt that correcting could inadvertently introduce error as well.

Plasma with EDTA was chosen as it was the cheapest commercial option available. Ethylenediaminetetraacetic acid (EDTA) chelates silver [20], and its presence in plasma theoretically could help extract Ag from the specimen contained within the dialysis tube. However, chelation of Ag into stable complexes also might hinder analyses, although measuring of excreted EDTA-metal complexes before and during chelation therapy has been described [20,21]. In addition, nitric acid digestion has been used to separate heavy metals from EDTA

191 [22]. However, depending on the drug for which the elution is performed a different anti-
192 coagulation agent might be preferred.
193 We used a 1:2 ratio of AgNP:poloxamer in this study to maximize the Ag content while still
194 maintaining full gelatination properties of the poloxamer. In a prior elution study [4] a 1:4 ratio
195 of AgNP:poloxamer was used with full gelatination, whereas specimens did not fully gel in a 1:1
196 ratio in a different study [23] but did when we tested at a 1:2 ratio (unpublished data). The
197 specimen composition as well as methodology (a single, larger amount of elution fluid with
198 sampling as opposed to full exchange of smaller amounts of elution fluid) between this study and
199 the prior study might account for the earlier tapering of elution and the flattening of the curve.
200 The fluid exchange would increase the gradient and therefore drive migration of Ag across the
201 dialysis tube membrane. The fluid quantity was chosen to allow a model with a larger amount of
202 fluid without full exchange was chosen to allow continuous stirring, the ability to submerge a
203 large specimen, and for cost of plasma (~$2,400 per 500ml).
204 While aluminum foil was wrapped tightly around the opening of the dishes, fluid loss, in
205 addition to sampling loss due to warming of the fluid was possible. Aluminum foil was chosen as
206 the initial intent was to perform all sampling with UF probes in addition to direct sampling and
207 using snap-on or screw-on lids would have damaged the probes. The study was converted to
208 direct sampling only at the last minute due to concerns of probe functioning and continued
209 patency during the initial assembly, and fluid was already in the dishes. The evaporative fluid
210 loss could account for the increase in Ag concentration of the fluid at the end of the study. No
211 attempt was made to estimate fluid losses during the study, as measuring at each time point
212 would have necessitated interrupting the elution and manipulating the specimen and might cause
213 additional fluid loss. However, measuring the volume at 96 hours could have been performed.

Fluid loss during a continuous release elution model is a known limitation of the model chosen [19], however removing and replacing all fluid at each time point might artificially keep a larger gradient intact than a continuous model would. In addition, a continuous model might more closely resemble the *in vivo* decrease in gradient between the slow-release compound and its environment.

The concentration of Ag measured in the samples obtained both by UF probe and pipette sampling decreased over time. This could be explained by the stagnant nature of the fluids within the tube, although care was taken to sample from the lower most area of the fluid in case of sedimentation. Elution experiments ideally are performed with constant stirring [19], or agitation prior to sampling [11]. We opted to not agitate the tubes to keep the probes fully submerged at all times and given that the probe would remain in the same place *in vivo*. The tubes for pipette sampling were similarly not agitated to not introduce a variable between the two methods, and samples were obtained from the lowest area in the tube. The higher variation seen in samples obtained by UF probes is interesting and indicates that care must be taken with *in vivo* sampling to account for variability, for example by taking multiple samples.

Additional limitations could be inconsistency in mixing the specimens and solutions as well as sampling or measurement errors. We included 3 repeats of the elution study to minimize the effect of both inconsistency and sampling. Six repeat samples were used in the static sampling study, however inconsistency between the solutions could still exist.

Conclusions

Elution into plasma and UF probe sampling of Ag in plasma are possible. While use of DPBS might be more cost effective, plasma should be considered for *in vitro* silver elution studies due to difference in elution and recovery. Silver nanoparticles in poloxamer 407 might release more

slowly than other drugs that have been studied under similar conditions. Both UF probe sampling and direct sampling underestimate the actual Ag concentration in the fluid. Ultrafiltration probe sampling of Ag is possible and UF probes could be used to measure *in vivo* local tissue concentrations, but the possibility of variations in the sampled fluid should be kept in mind.

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312

313 # Supporting information

314 S1: Excel file with original data

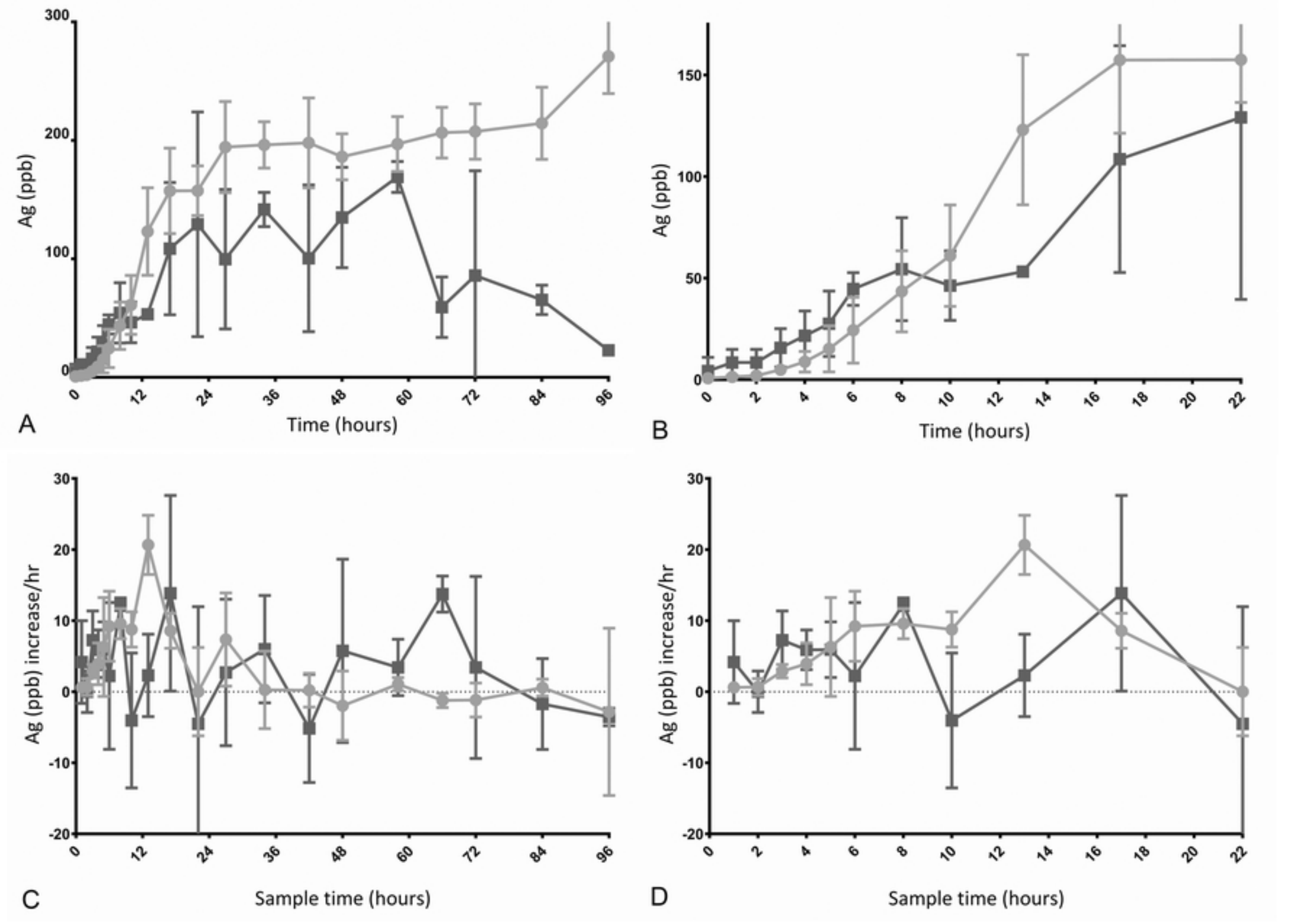
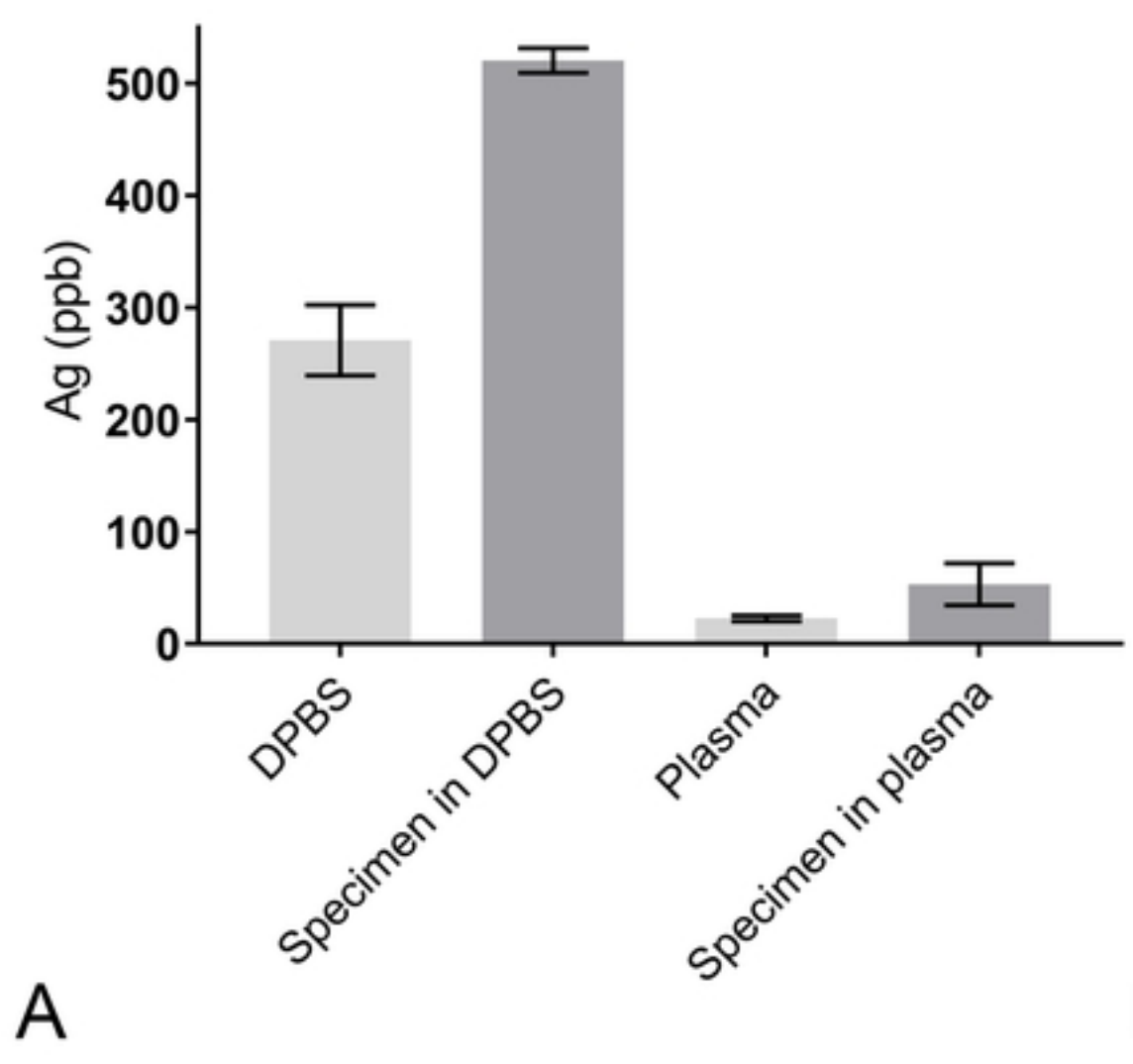


Figure 1ABCD


Ag (ppb)
500
400
300
200
100
0
DPBS
Specimen in DPBS
Plasma
Specimen in plasma
A

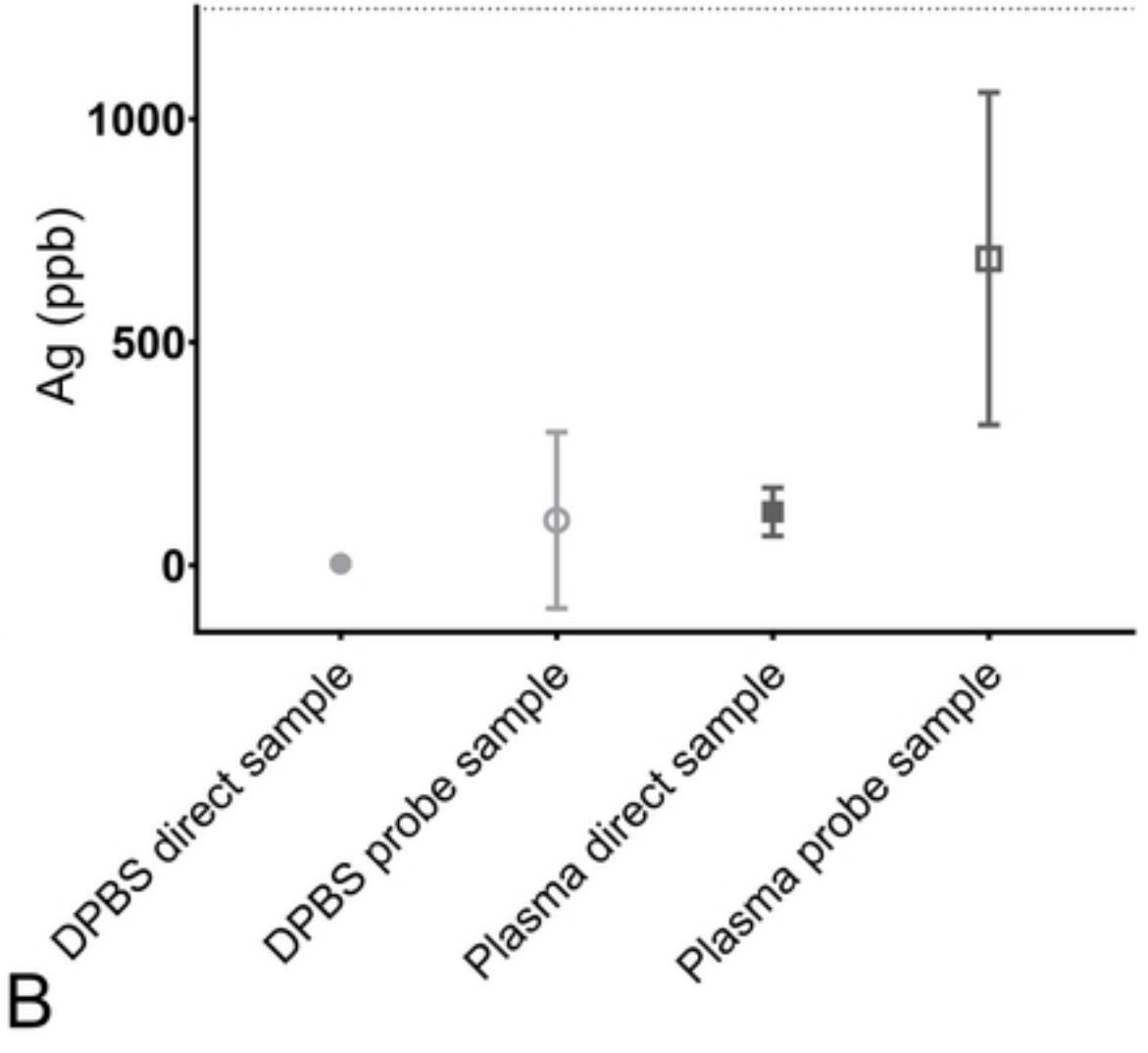

Ag (ppb)
1000
500
0
DPBS direct sample
DPBS probe sample
Plasma direct sample
Plasma probe sample
B

Figure 2AB