Sona Krizkova[1,2]
Eva Jilkova[1]
Ludmila Krejcova[1]
Natalia Cernei[1]
David Hynek[1,2]
Branislav Ruttkay-Nedecky[1,2]
Jiri Sochor[1,2]
Jindrich Kynicky[2,3]
Vojtech Adam[1,2]
Rene Kizek[1,2]

[1]Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University in Brno, Brno, Czech Republic
[2]Central European Institute of Technology, Brno University of Technology, Brno, Czech Republic
[3]Department of Geology and Pedology, Faculty of Forestry and Wood Technology, Mendel University in Brno, Brno, Czech Republic

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Research Article

Rapid superparamagnetic-beads-based automated immunoseparation of Zn-proteins from *Staphylococcus aureus* with nanogram yield

Pathogenic bacteria have become a serious socio-economic concern. Immunomagnetic separation-based methods create new possibilities for rapidly recognizing many of these pathogens. The aim of this study was to use superparamagnetic particles-based fully automated instrumentation to isolate pathogen *Staphylococcus aureus* and its Zn(II) containing proteins (Zn-proteins). The isolated bacteria were immediately purified and disintegrated prior to immunoextraction of Zn-proteins by superparamagnetic beads modified with chicken anti-Zn(II) antibody. *S. aureus* culture was treated with $ZnCl_2$. Optimal pathogen isolation and subsequent disintegration assay steps were carried out with minimal handling. (i) Optimization of bacteria capturing: Superparamagnetic microparticles composed of human IgG were used as the binding surface for acquiring live *S. aureus*. The effect of antibodies concentration, ionic strength, and incubation time was concurrently investigated. (ii) Optimization of zinc proteins isolation: pure and intact bacteria isolated by the optimized method were sonicated. The extracts obtained were subsequently analyzed using superparamagnetic particles modified with chicken antibody against zinc(II) ions. (iii) Moreover, various types of bacterial zinc(II) proteins precipitations from particle–surface interactions were tested and associated protein profiles were identified using SDS-PAGE. Use of a robotic pipetting system sped up sample preparation to less than 4 h. Cell lysis and Zn-protein extractions were obtained from a minimum of 100 cells with sufficient yield for SDS-PAGE (tens ng of proteins). Zn(II) content and cell count in the extracts increased exponentially. Furthermore, Zn(II) and proteins balances were determined in cell lysate, extract, and retentate.

Keywords:
Bacteria / Immunoseparation / Robotic system / Superparamagnetic particles / Zinc(II) / proteins
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1 Introduction

Staphylococcus aureus has become a looming threat due to its increasingly multiple drug resistance to many antibiotics. In 1947, four years after mass production of penicillin began, *S. aureus* penicillin-resistant strains were identified [1]. In 1959, methicillin, a new antibiotic, was found to be effective against penicillin-resistant staphylococci. The bacteria eventually became resistant to methicillin that consequently led to the discovery of the first methicillin-resistant strain of *S. aureus* (MRSA) in 1961. Currently, approximately 65% of staphylococcal strains have been identified with MRSA phenotype (resistance to beta-lactam antibiotics and the cephalosporins) [2]. Emergence and the increased number of antibiotic-resistant bacteria have resulted in exploring alternatives to antibiotics such as heavy-metal complexes [3–7], heavy-metal nanomaterials and nanoparticles [8].

Heavy metals, namely zinc-based complexes seem to be promising antibacterial agents; however, bacterial pathogens are able to develop or acquire resistance to heavy metals. In 1991, loss of methicillin resistance due to elimination of resistance to heavy metals and tetracycline was reported [9]. Studies found that exposure to nutraceutics, namely zinc derivatives, echinacea and garlic products exhibited resistance to antibiotics in *S. aureus* [10]. These investigations suggested a strong association between methicillin resistance and zinc [11]. Moodley et al. demonstrated that feed supplemented with tetracycline or zinc increased the number of MRSA in

Correspondence: Dr. Rene Kizek, Department of Chemistry and Biochemistry, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic
E-mail: kizek@sci.muni.cz
Fax: +420-5-4521-2044

Abbreviations: DPV, differential pulse voltammetry; **HRP**, horseradish peroxidase; **MRSA**, methicillin-resistant strain of *Staphylococcus aureus*

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the nasal cavity of pigs [12]. It was discovered that gene *czrC* encoding zinc and cadmium resistance in MRSA was related to methicillin resistance [13]. This gene is widespread both in humans and animals and is predicted to encode transporters, metal-carrying proteins. Zinc-dependent endopeptidases, specifically resistance factor HmrA from MRSA, have been found to belong to a group of proteins characterized with zinc and antibiotic resistance [14]. HmrA, an antibiotic resistance factor of methicillin-resistant *S. aureus*, is common among bacterial species. Two zinc-dependent metalloproteases, ZmpA and ZmpB, have been shown to influence the resistance of *Burkholderia cenocepacia* to host antimicrobial peptides [15]. In addition, it was discovered that a fusion protein with a penicillin-binding site and zinc-binding metalloproteinase domain is involved in signal transferring in staphylococcal resistance to beta-lactam antibiotics [16]. Associations between types and levels of heavy metals and specific patterns of antibiotics resistance share cross-resistance. Several mechanisms underlie this process and are most important due to their nonspecific properties [17]. With the exception of the previously mentioned associations with multiple antibiotic resistance, the following zinc-bound proteins possess staphylococcal pathogenic properties: superantigens [18, 19], lysostafin [20], and other extracellular proteases [21], enterotoxins [22], adhesion factors [23], transporter proteins [24], biofilm formation [25], transcription factors, and DNA-binding proteins [26, 27].

Particle-based proteins extraction is a novel and simple process employing (super)paramagnetic beads coupled with specific antibody, ligand protein, or low-molecular-mass ligand. There are several protocols for antibody immobilization, proteins, organelles, or whole cells. Similar to other immunochemical assays, beads-based protocols maximize optimization and give reliable results [28, 29] as bead-based sensors for pesticides [30], toxins [31], viruses [32], and other pathogens [33, 34], biomarkers [35, 36], detection or separation of rare cells [37–39], or cells sorting and counting [40]. Heavy-metal immunoassays utilized anti-heavy-metal antibodies that monitored binding [41] environment pollutants, Pb(II) [42], Hg(II) [43], As(IV, V), Cd(II) [44], Cu(II) [45], U(VI) [46], and rare heavy metals Ru(II) [47] and In(III) [48]. Uranyl-specific antibodies suitable for cellular imaging were developed [49] based on these studies. Antibodies against Zn(II) were successfully prepared in previous investigations [50]. Except Krizkova et al. [51], who immunoextracted zinc-binding proteins from human plasma, no articles about using of antibodies for separation of heavy metal-bound proteins have been published. Various techniques such as sucrose density gradient centrifugation [52], polyethylene glycol precipitation [53], electrophoresis [54–56], chromatography [57–59] and ultrafiltration [60] have been employed for the separation of metalloproteins from biological sample. However, many of them require high sample volume or are not applicable for real sample analysis.

Magnetic bead-based analytical procedures can be miniaturized, are broadly applicable and widely applied in lab-on-chip and robotic sample handling systems [61]. They provide high sensitivity and specificity. The objective of this study was to explore automatic immune-superparamagnetic-bead-based methods for cell isolation and techniques for specific protein group separations. Zn(II) binding proteins were extracted from *S. aureus* via specific anti-Zn(II) antibody with minimal handling. The comprehensive isolation scheme is outlined in Fig. 1.

2 Material and methods

2.1 Chemicals and water purification

Unless otherwise indicated, all chemicals were purchased from Sigma Aldrich (USA) and met or exceeded ACS specifications. Deionized water underwent demineralization by reverse osmosis using Aqua Osmotic 02 instruments (Aqua Osmotic, Tisnov, Czech Republic) and was subsequently purified using Millipore RG (Millipore, USA, 18 MΩ)–MilliQ water.

2.2 Cultivation and monitoring of bacterial growth

S. aureus cultures (NCTC 8511, Czech Collection of Microorganisms, Brno, Czech Republic) were stored on agar slants (meat-peptone agar No. 1, MPA 1). Cultivation was performed in liquid broth (meat peptone 5 g/L, NaCl 5 g/L, beef extract 1.5 g/L, yeast extract 1.5 g/L, pH 7.4 ± 0.2 HIMEDIA, India). Bacteria were cultivated at 37°C with shaking under 600 rpm using Incubator Hood TH 15 (Edmund Buhler, Germany) for 6 h, until the optical density of the culture reached values of 0.1 at 600 nm (Specord 210, Analytik Jena, United Kingdom). Grown culture with this optical density was used in the proceeding experiments. For monitoring bacterial growth in the presence of Zn(II), 100 mL of the bacterial culture was spiked with 0, 1.5, 5, 10, 25, 50, 100, and 250 μM $ZnCl_2$. The solution was transferred into 3 mL plastic cuvettes (Analytik Jena), covered with lids, and turbidity measurements were taken at 600 nm every 30 min for 24 h in a thermostated spectrophotometer (Specord 210, Analytik Jena). Five replicates were performed for each sample. For immunoseparation of staphylococci using Zn-proteins, 25 mL of bacterial culture was spiked with 100 μM $ZnCl_2$ and cultivated for an additional 6 h.

2.3 Superparamagnetic beads modification

IgG from human serum (#I4506) were purchased from Sigma-Aldrich. Chicken antibodies were prepared by HENA, Prague, Czech Republic. Two hens were immunized with Zn-KLH (keyhole limpet haemocyanin) complex. IgY fraction reactive to Zn-KLH was obtained from egg yolk. The antibodies were stabilized with 0.1% NaN_3 in PBS and protein concentration was 39.6 mg/mL in immunoglobulin fraction.

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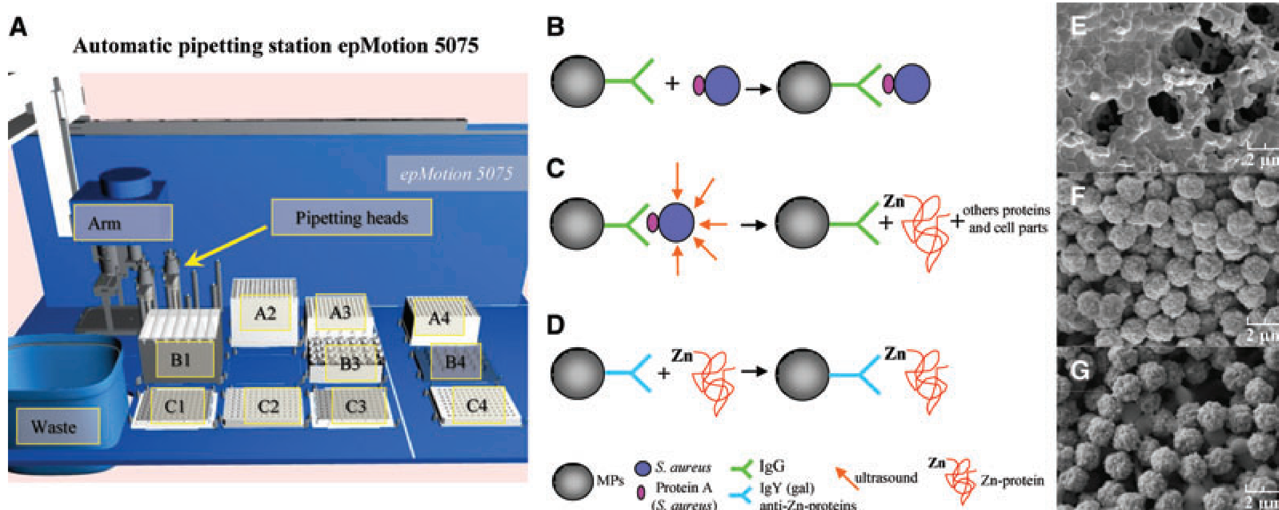

Figure 1. Schematic illustration of the immunoextraction process: (A) Scheme of automatic pipetting robotic station epMotion 5075: A2, A3, A4 position on tips, B1 dispenser with washing solutions, and B2 waste, B3 dispenser for antibodies and substrate, B4 magnetic stand, C1 thermostated position (4°C), C2 a C4 manipulation positions, C3 thermostated position (37°C). (B) immunocapture of staphylococcal cells to superparamagnetic beads (MPs) modified with human IgG via staphylococcal surface protein A; (C) cells' lysis with ultrasound; (D) immunoextraction of Zn-proteins from bacterial lysate with superparamagnetic beads modified by chicken anti-Zn antibodies. SEM image of (E) *S. aureus*; (F) superparamagnetic beads modified with human IgG; (G) *S. aureus* cells captured to IgG-modified superparamagnetic beads.

For covalent antibody immobilization, *p*-toluenesulphonyl chloride-activated superparamagnetic polystyrene beads coated with polyurethane layer were used (Dynabeads® MyOne™ Tosyl activated, #655.01). Antibody preparation and immobilization protocol was adapted from the supplier's manual (Invitrogen, Norway). For immobilization, 1000 µg of the antibodies per 25 mg of beads were used. Prior to immobilization, NaN_3 was removed and antibodies were acidified to pH 2.5 by addition of HCl. After 15 min, the antibodies were brought into physiological pH (7.4). For all buffer exchanges, Amicon Ultra 0.5 columns with membrane cut off 50 K (Millipore, Denmark) were used. Covalent immobilization was carried out in total volume of 625 µL in the presence of 0.1 M borate buffer of pH 9.5 with 0.1 M $(NH_4)_2SO_4$ for 24 h under mild rotation. The free particles surface was then blocked with 0.5% BSA in PBS w/v and 0.05% Tween-20 v/v for 10 h. After blocking, the beads were washed three times with 1 mL of 0.1% BSA in PBS w/v with 0.05% Tween-20 v/v and resuspended in 625 µL of storage buffer (washing buffer with 0.02% NaN_3 w/v).

Functionality of the beads with chicken anti-Zn antibody was tested by using rabbit anti-chicken/horseradish peroxidase conjugate and tetramethylbenzidine as a chromogenic substrate. The absorbance was read in microplates at 450 nm (Original MultiskanEx, Thermo Electron, USA). Beads coated with human IgG were tested using experimental immunoseparation of bacteria from culture spiked with 100 µM $ZnCl_2$. Cultivation was performed for 24 h at 37°C with shaking at 540 rpm prior to turbidity measurements (600 nm). Measurements were taken every 30 min in a thermostated microplate reader (Original MultiskanEx, Thermo Electron Corporation) and managed with Ascent 2.6 software (Thermo Scientific, USA). For immunoextractions, 5 mg/mL of beads per 500 µL of culture were used. Immunoextraction was performed on a rotating programmable rotator-mixer (Biosan, Latvia) in 2 mL microtubes for 1 h. The beads were then separated from the solution, washed with 250 µL of the medium and inoculated into 250 µL of medium in a microplate (Nunc, Germany).

2.4 Immunocapturing of bacteria and Zn-proteins

Sample handling prior to electrochemical analysis involved an automated pipetting station Ep-Motion 5075 (Eppendorf, Hamburg, Germany) with computer control (Fig. 1A). Positions C1 and C4 were thermostated (Epthermoadapter PCR96). The samples can be placed in position B3 Ep 0.5/1.5/2 mL adaptor. In B1 position, module reservoirs for washing solutions and waste were placed. Tips were placed in positions A4 (ePtips 50), A3 (ePtips 300), and A2 (ePtips 1000). Transfer was ensured by a robotic arm with pipetting adaptors (TS50, TS300, TS1000–numeric labeling refers to maximal pipetting volume in microliters) and a gripper for platforms transport (TG-T). The program sequence was edited and the station was controlled in pEditor 4.0. For sample preparation, two platforms were used: Thermorack for 24 × 1.5–2 mL microtubes (Position C3), used for storage of working solutions and 96-well DPW plate with well volume of 1000 µL (Position C1) which was thermostated. After immunoseparation, the magnetic particles were attracted by a Promega magnetic pad (Promega, USA; position B4) and the solutions were transferred to a new DPW well. The samples

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were then used for electrochemical Zn(II) determination and SDS-PAGE analysis.

The following procedure and parameters were used for immunoextraction: 10 μL of the IgG-modified superparamagnetic beads per one sample were pipetted into each single well. After washing the beads with 3 × 270 μL of broth, different volumes (0, 1, 10, 25, 100, 200, 300, and 400 μL) of staphylococcal bacterial culture grown in $ZnCl_2$ were pipetted onto the beads and filled with broth to a final volume of 500 μL (Fig. 1B). After 1 h of cultivation, the solution was removed and beads with captured bacteria were washed with 3 × 500 μL of broth, transferred into new wells, refilled with an additional 200 μL of broth and further cultivated at 37°C for 3 h. After enrichment cultivation, ultrasound was used to lyse bacteria (450 Hz, 2 min, Bandelin, Germany), Fig. 1C. Thereafter (Fig. 1D), 10 μL of anti-ZnIgY-modified superparamagnetic beads per one sample/well were pipetted and washed with 300 μL of PBS (pH 4.5). Bacterial lysate (150 μL) was added and after 30 min in incubation and mix by pipetting, the solution was removed and the beads were washed with 500 μL PBS (pH 7.5). Bound Zn-proteins were eluted into 30 μL of 0.1 M citrate, pH 2.5. Elution was repeated three times.

2.5 Electrochemical analyses

2.5.1 Zn(II) determination

Prior to Zn(II) determination, the samples were digested using microwave heating [62–64]. The mineralization of samples briefly took place in a microwave system, Multiwave3000 (Anton-Paar, Graz, Austria). A sample (15 μL) was placed into MG5 glass vials and 350 μL of nitric acid (65%, w/w) and 150 μL of hydrogen peroxide (30%, w/w) were added. Prepared samples were sealed and placed into a 64MG5 rotor (Anton-Paar). The rotor with the samples was inserted into the microwave system and the microwave digestion was carried out under the following conditions: power 50 W for 10 min, power 100 W for 30 min, cooling (power 0 W) for 10 min, maximum temperature 80°C. Following sample preparation, subsequent electrochemical measurements were used: 100 μL mineralized sample was pipetted into Eppendorf tubes with 900 μL of 0.2 M acetate buffer (pH 5.00). A blank digestion was simultaneously carried out in the same way.

Differential pulse voltammetry (DPV) was performed on 797 VA Stand instrument connected to 813 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes. A hanging mercury drop electrode with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and the platinum was the auxiliary electrode. For data processing, VA Database 2.2 by Metrohm was employed. The analyzed samples were deoxygenated prior to measurement by purging with argon (99.999%). Acetate buffer (0.2 M CH_3COONa + CH_3COOH, pH 5) was used as a supporting electrolyte. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as follows: initial potential of −1.3 V, end potential of 0.15 V, deoxygenating with argon for 90 s, deposition time of 120 s, time interval of 0.04 s, step potential of 4 mV, modulation amplitude of 25 mV, adsorption potential of −1.15 V, volume of injected sample–15 μL, volume of measurement cell–2 mL (15 μL of sample + 1985 μL of acetate buffer) [63].

2.5.2 Metallothionein determination

The obtained cells were washed three times with phosphate buffer of pH 7 and weighed. The samples (approximately 0.2 g of fresh weight) were transferred to test tubes (2 mL) (Eppendorf) and liquid nitrogen was added. The samples were frozen to disrupt the cells. The mixture was prepared using an ULTRA-TURRAX T8 hand-operated homogenizer (IKA, Germany) at 25 000 rpm for 3 min. The homogenate was transferred to a new test tube and vortexed for 15 min at 4°C (Vortex Genie). The supernatant was subsequently heat treated. The sample was kept at 99°C in a thermomixer (Eppendorf) for 15 min with occasional stirring, and then cooled to 4°C. The denatured homogenates were centrifuged at 4°C, 15 000 rpm for 30 min (Eppendorf 5402). Heat treatment effectively denatured and removed the high-molecular-weight proteins [65]. DPV with Brdicka reaction was used to analyze processed samples. The data were interpreted using a 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland); it used a standard cell with three electrodes and cooled sample holder (4°C) according to protocol by Fabrik et al. [66]. A hanging mercury drop electrode with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and glassy carbon was the auxiliary electrode. GPES 4.9 supplied by software EcoChemie was employed for smoothing and baseline corrections of the obtained data. A supernatant sample (200 μL) was pipetted into the electrochemical cell containing 1800 μL of Brdicka supporting electrolyte and measured using DPV. The electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer ($NH_3(aq)$ + NH_4Cl, pH 9.6) was used and changed per one analysis. DPV parameters were: initial potential of −0.7 V, end potential of −1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential of 2 mV, modulation amplitude of −250 mV, $E_{ads} = 0$ V. All experiments were carried out at constant temperature of 4°C (Julabo F12 cooler).

2.6 Total protein content determination

The obtained cells were washed three times with 0.2 M phosphate buffer of pH 7. Weighed bacterial samples (approximately 0.1 g of fresh weight) were transferred to test tubes (2 mL) (Eppendorf), and liquid nitrogen was added. The samples were frozen to disrupt the cells. The mixture was prepared using a hand-operated ULTRA-TURRAX T8 homogenizer (IKA) at 25 000 rpm for 3 min. The homogenate

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was transferred to a new test tube. The mixture was further homogenized by shaking using a Vortex-2 Genie (Scientific Industries, USA) at 4°C for 30 min. The homogenate was centrifuged (14 000 rpm) for 30 min at 4°C using Universal 32 R centrifuge (Hettich-Zentrifugen, Tuttlingen, Germany). Prior to analysis, the supernatant was filtered through a membrane filter (0.45 μm Nylon filter disk, Millipore).

Total proteins content was determined using Lowry method [67]. Briefly, 180 μL of R_1 reagent (7 mM Na-K tartrate, 0.81 M sodium carbonate, 0.5 M NaOH) was mixed with 200 μL of the sample and vortexed for 10 s (Vortex-2 Genie, Scientific Industries) at 1200 rpm. Afterwards, the sample was incubated for 10 min at 55°C. After cooling to 20°C, 20 μL of R_2 (70 mM Na-K tartrate, 40 mM $CuSO_4$) reagent was added followed by vortex and addition of 600 μL of reagent R_3 (37.5 μL Folin-Ciocalteau reagent diluted with 562.5 μL water). Subsequent vortexing and incubation for 10 min took place at 55°C followed by absorbance measurements at $\lambda = 640$ nm using SPEKOL 210 (Analytik Jena, Germany).

2.7 SDS-PAGE

Electrophoresis was performed using a mini protean tetra apparatus with gel dimensions of 8.3 × 7.3 cm (Bio-Rad, USA). First 15% or 12.5% w/v running, then 5% w/v stacking gel was poured. The gels were prepared from 30% m/v acrylamide stock solution with 1% w/v bisacrylamide. The polymerization of the running or stacking gels was carried out at room temperature for 45 min. Prior to analysis, the samples were mixed with nonreducing sample buffer in a 2:1 ratio. The samples were incubated at 93°C for 3 min and the sample was loaded onto a gel. In order to compare proteins content and composition before and after immunoextraction, 10 μL of bacterial lysate from 270 × 10^3 cells (e.g. 300 ng of total protein) and corresponding retentate was loaded. “Precision plus protein standards” protein ladder from Bio-Rad was used to determine molecular mass. The electrophoresis was run at 150 V for 1 h at 23°C (Power Basic, Bio-Rad) in tris-glycine buffer (0.025 M Trizma-base, 0.19 M glycine and 3.5 mM SDS, pH 8.3). Then the gels were stained with Coomassie blue and consequently with silver (in case the protein concentration in the samples was below detection limit of Coomassie blue staining). The procedure of rapid Coomassie blue staining was adopted from Wong et al. [68] and silver staining was performed according to Krizkova et al. [69] but the fixation (1.1% v/v acetic acid, 6.4% v/v methanol, and 0.37% v/v formaldehyde) and first two washing steps (50% v/v methanol) were omitted.

2.8 Western blotting

After electrophoretic separation, the proteins (200 ng of eluate and 50 ng of anti-Zn antibody) were transferred on a PVDF membrane using Biometra Fastblot apparatus (Biometra, Germany). PVDF membranes were activated by soaking in methanol for 30 s prior to blotting. The membrane was then equilibrated for 5 min in blotting buffer (12.5 mM Tris-base, 75 mM glycine, and 15% v/v methanol). The blotting sandwich was composed of three layers of filter paper soaked in blotting buffer, membrane and polyacrylamide gel, and three layers of soaked filter paper. The blotting was carried out for 1 h at a constant current of 0.9 mA for 1 cm^2 of the membrane. After the transfer, the membrane was blocked in 1% BSA PBS (137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4, and 4.3 mM Na_2HPO_4, pH 7.4) for 30 min. The incubation with secondary antibody (rabbit anti-chicken/horseradish peroxidase conjugate; Sigma-Aldrich; in dilution 1:6000) was performed for 1 h at room temperature. The membrane was then washed three times with PBS-T for 5 min and incubated with chromogenic substrate (0.4 mg/mL 3-aminoethyl-9-carbazole in 0.5 M acetate buffer with 0.1% H_2O_2, pH 5.5). After adequate development, the reaction was stopped by rinsing with water.

2.9 SEM

A modern scanning electron microscope with motorized stage, full software control, and image acquisition was recognized as a relatively easy way for automated high-resolution documentation of *S. aureus* samples. Bacteria sample filtration was carried out using Millipore syringe filters (0.45 and 0.2 microns) (Millipore). The fixation was successfully done by persistent Nd-magnet sections. The fixation was gone through additional washing process with washing solution (a sterile solution of sodium chloride). Only maximum, precise prepared samples were fixed in solution by persistent Sm-magnet during the two dehydration steps. These steps were performed in graded ethanol bathes (70 and 100%, each for 2 min) before being coated with gold [70]. Samples were coated with 5 nm of gold to prevent sample charging. A gold coater Quorum Technologies K950X was used. For each experiment, three independent samples of *S. aureus* on different tablet sections (glass, pure Si, and Millipore syringe filters) were documented. FEG-SEM TESCAN MIRA 3 XMU was used for documentation. This model is equipped with a high brightness Schottky field emitter for low noise imaging at fast scanning rates. The SEM was fitted with Everhart-Thronley type of SE detector, high-speed YAG scintillator-based BSE detector and panchromatic CL detector.

3 Results and discussion

3.1 Bacterial growth in the presence of Zn(II)

Staphylococci were grown in a medium containing various $ZnCl_2$ concentrations (0, 1.5, 5, 10, 25, 50, 100, and 250 μM, Fig. 2A). Bacterial growth was observed at a slower rate in the presence of the lowest dose of Zn(II) (1.5 μM $ZnCl_2$) than comparable controls. Changes in bacterial growth were observed during the first 2 h of cultivation. However, after

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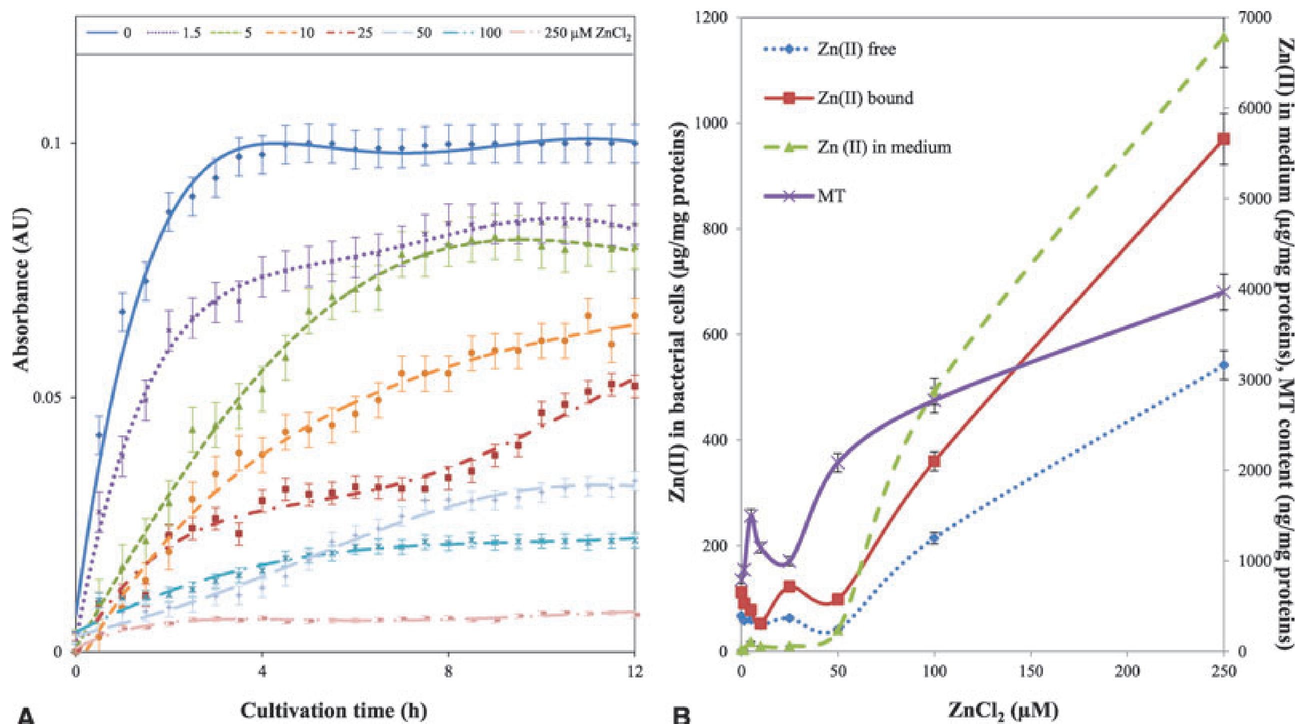

Figure 2. (A) Growth curves of *S. aureus* treated with various concentrations of $ZnCl_2$ (0–250 μM). (B) Metallothionein and Zn(II) content in *S. aureus* cells cultivated for 12 h in the presence of $ZnCl_2$ and content of remaining Zn(II) in medium.

12 h, the $ZnCl_2$ concentrations above 25 μM caused a 50% decrease in turbidity in comparison to the control (0 μM $ZnCl_2$). The observed changes may be due to Zn(II) or chloride. It is well known that *S. aureus* is halophilic. The medium contained 5 g/L of NaCl, e.g. 0.125 M of chloride ions. At the highest $ZnCl_2$ concentration, the change in chloride concentration was 0.4%; thus the growth of bacteria would not be affected by chloride anion [71] in contrast to water-soluble zinc salts [72, 73]. The observed changes in bacterial growth are in agreement with published results showing $ZnCl_2$ and other Zn(II) compounds can be effective antibacterial agents [74, 75].

3.2 Metallothionein and Zn(II) determination

In all experimental groups, metallothionein content was expressed as ng per mg of proteins with increasing concentrations of Zn(II) ions in the treated bacteria. The average increase of MT content was 1.44-fold within a 1.5 to 25 μM $ZnCl_2$ range. At higher concentrations of $ZnCl_2$–50, 100, and 250 μM–the increase was 2.7-, 3.5-, and 5-fold, respectively (Fig. 2B). The results indicate that bacteria synthesized metallothionein acts as a protective agent against Zn(II) adverse actions. The role of metallothioneins in bacterial resistance to zinc and other heavy metals was published and reviewed previously [76, 77]. Brdicka reaction was successfully employed for metallothionein determination in staphylococci exposed to $CdCl_2$ [78].

Total Zn(II) content in cells may be subdivided into the free and bound form, which corresponds to its ligand environment. Free Zn(II) is chelated by low-molecular compounds like thiols or enzyme cofactors. Bound Zn(II) is chelated by high-molecular compounds, such as proteins, as well as DNA or polysaccharides. Free Zn(II) content related to total protein content was constant at $ZnCl_2$ concentration lower than 50 μM with 3.6- and 9-fold increase at 100 and 250 μM $ZnCl_2$, respectively (Fig. 2B). Bound Zn(II) content related to total protein content at $ZnCl_2$ concentrations lower than 10 μM was slightly decreased as compared to control. At 25 and 50 μM $ZnCl_2$, the content of bound Zn(II) was comparable to control, but at 100 and 250 μM, $ZnCl_2$ 3.2- and 8.7-fold increases were observed. Zn(II) concentration in medium related to bacterial growth expressed as proteins content was constant at $ZnCl_2$ concentrations lower than 50 μM (Fig. 2B). At 50 μM $ZnCl_2$, 12-fold increase in Zn(II) content in medium was observed, but at 100 and 250 μM $ZnCl_2$, Zn(II) content in medium increased 140- and 330-fold, respectively (Fig. 2B). The obtained results indicate that bacteria were able to withstand $ZnCl_2$ concentrations lower than 50 μM either via activation of Zn(II) transport mechanisms or protective compounds synthesis. Based on the presented data, it can be concluded that $ZnCl_2$ concentrations higher than 25 μM resulted in incontrollable intake of Zn(II) due to massive differences in the extracellular and intracellular Zn(II) concentrations. This was partially balanced by MT synthesis, but at $ZnCl_2$ concentration above 50 μM, the bacteria were unable to regulate

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Zn(II) intake. These values, along with dramatic decrease in bacterial growth observed at Zn(II) concentrations above 50 μM, are consistent with stated growth curves . Compared to previous publications, which found that *S. aureus* exhibited minimal inhibition concentrations with a range of 0.25 to 2 mM $ZnCl_2$ [74], current results indicate lower minimal inhibition concentrations values. The slight discrepancy may be due to limited solubility of higher $ZnCl_2$ concentrations in solid agar plates.

3.3 Immunoseparation of bacterial cells

Two types of antibody-modified superparamagnetic beads were prepared for immunoseparation of staphylococcal cells and their Zn-proteins components. Scheme of the immunoextraction process is shown in Fig. 1: (B) immunocapture of staphylococcal cells to superparamagnetic beads (MPs) modified with human IgG via staphylococcal surface protein A, (C) cells' lyses with ultrasound, (D) immunoextraction of Zn-proteins from bacterial lysate with superparamagnetic beads modified by chicken anti-Zn antibodies. The electron microphotographies of *S. aureus*, superparamagnetic beads modified with human IgG and *S. aureus* cells captured IgG-modified superparamagnetic beads are shown in Fig. 1E, F, and G.

Staphylococcal superantigen surface protein A was used for immunoseparation since it is known to bind to nearly all types of mammalian immunoglobulins. Commercially available human IgG were covalently immobilized on the surface of the beads. The performance of the immunoseparation system was tested with bacteria of varying culture (Fig. 3A). Results indicate that bacteria were successfully separated, transferred into new medium, and able to grow. Bacterial growth was recorded by continuous turbidity monitoring. Evidence of bacteria binding to beads was also evident by SEM (Fig. 3B). This system was found to be reliable, selective, and specific for *Staphylococcus* and G-group *Streptococcus* species even in bacterial mixtures [79]. Higher specificity could be achieved using specific antibodies. However, because the aim of this study was to design an immunoextraction cell model, a low-cost and verified alternative was used.

Immobilization of chicken anti-Zn(II) antibody was tested by secondary rabbit anti-chicken/horseradish peroxidase (HRP) conjugate and chromogenic substrate tetramethylbenzidine; captured proteins to primary antibodies and their elution with pH changes were tested. Compared to rabbit anti-mouse/HRP conjugate control, increased absorbance at 450 nm showed both beads and eluate were present (Fig. 3C). Zn(II) metals are loosely bound to proteins through unspecific interactions with amino acid residues such as cysteine, methionine, and histidine or they interact with specific motifs within zinc-fingers or enzyme-active sites [80]. Since the strength of Zn(II)-protein interaction plays a critical role in immunoextraction, the proposed immunoextraction method was tested using BSA since it is a nonspecific Zn(II) chelator. Using anti-Zn-antibody-modified beads, 125 ng of BSA were extracted (Fig. 3D). SDS-PAGE gel stained with silver (detection limit in order of units of ng) showed no proteins were extracted using human IgG-modified beads (Fig. 3D).

IgG-modified beads successfully separated 700 bacterial cells from a volume of 1 mL within 30 min. The growth of bacteria was monitored by continual measurement of turbidity. Based on the results shown in Fig. 4A during the 300-min growth period, the turbidity increased parallel to control in which no bacteria occurred. The achieved detection limit is sufficient for standard microbiological applications similar to monitoring bacterial contamination in food. The measured detection limit–700 cells/mL–corresponded to 100 CFU/mL as determined by plate counting, while less than 300 CFU/mL of *S. aureus* is tolerated in crude cow milk. Detected *S. aureus* count is also expected in 50 mL of sea water basin after one day of standard use. While traditional immunological methods can often take days to assess and are time consuming, the current method enables the detection of cells within 1.5 h.

3.4 Immunoseparation of zinc-containing-proteins from bacterial cells

Immunomagnetic separation combined with sensitive methods such as mass spectroscopy [81], PCR [82], or bioluminescence [83] are commonly used for rapid detection of pathogenic bacteria. Immunomagnetic separation method can be automated, miniaturized, and its performance capabilities are comparable with microfluidic technology. The current innovative procedure is unique in that its use is compatible with common microplate readers. Use of human IgG-modified beads allows the separation and detection of *Staphylococcus* and G-group *Streptococcus* ssp.; however, detection specificity could be further improved by using specific antibody or selective medium. The combination of immunomagnetic separation without the need for sophisticated instrumentation is very promising and offers great potential for wide field applications, especially in developing countries, as suggested in Liandris et al., where antibody-coated superparamagnetic beads were used to separate and detect *Mycobacterium* ssp. using quantum dot-labelled antibody and UV transluminator [84].

Immunoextraction of Zn-proteins from ultrasound-lyzed bacteria, cultivated in 100 μM $ZnCl_2$ for 12 h, were tested. These conditions were chosen based on results shown in Section 3.1. SDS-PAGE of bacterial lysate before and after immunoextraction is shown in Fig. 4B. Results show some proteins were removed, while some were retained. The proteins were eluted with 0.1 M citrate at pH 2.5. Source of proteins presented in eluate were either bacterial proteins or chicken antibodies, released from beads' surfaces, acting as artifacts. To distinguish the sources of proteins, a Western blotting test with rabbit anti-chicken/HRP conjugate was implemented. While the chicken antibody separated under reducing conditions, exhibiting three bands

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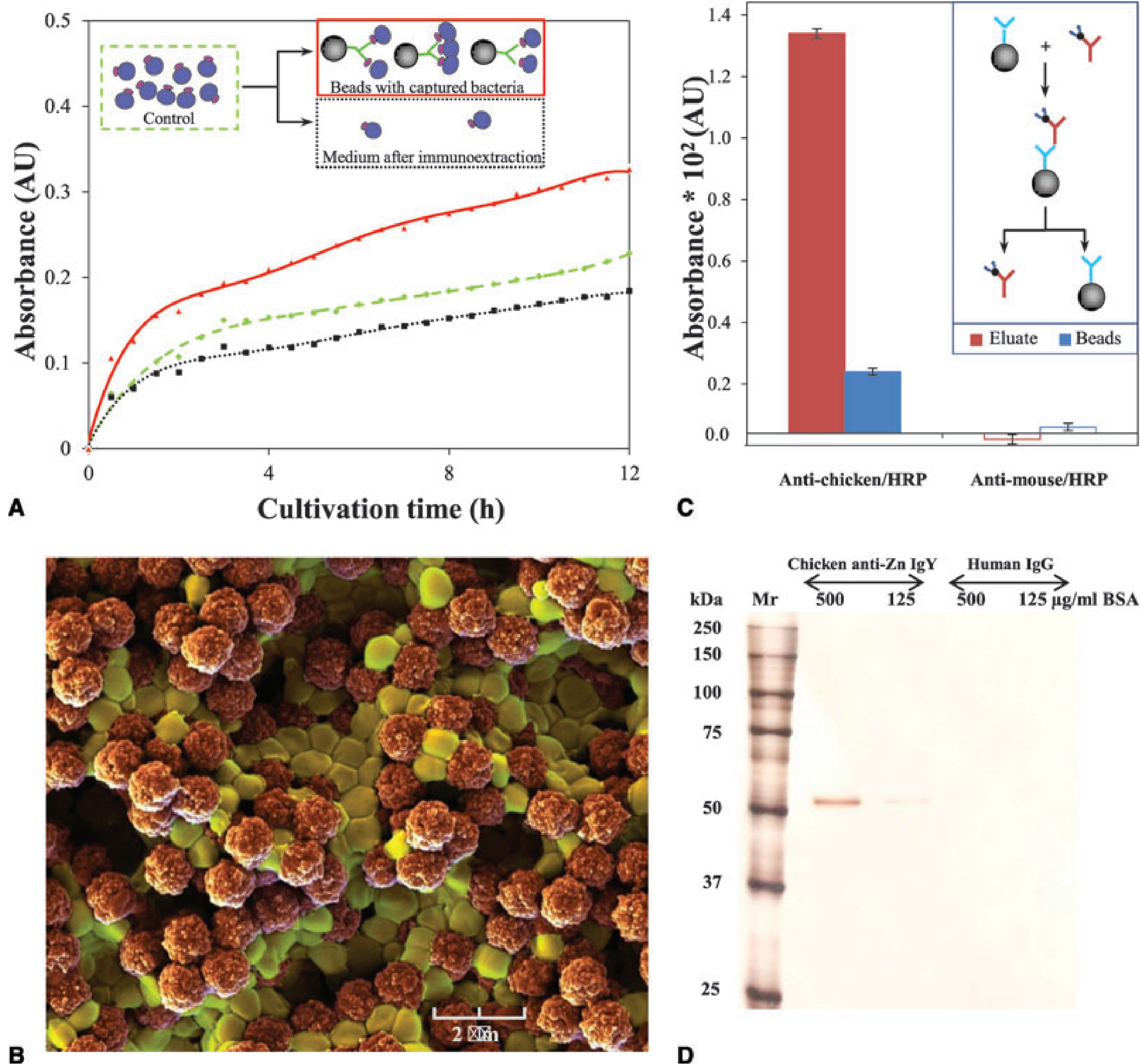

Figure 3. Verification of antibodies immobilization to superparamagnetic beads. (A) Growth of *S. aureus* immunoextracted from medium compared to control (intact bacterial culture) and medium after immunoextraction. Dashed line: control, full line: beads with captured bacteria, dotted line: medium after immunoextraction. (B) SEM image of *S. aureus* captured to the beads. (C) Verification of chicken IgG immobilization to the beads with secondary rabbit anti-chicken/HRP conjugate. Red column: eluate, blue column: beads. Full columns: rabbit anti-chicken antibodies, empty columns: rabbit anti-mouse antibodies; (D) immunoextraction of BSA (model Zn-protein).

corresponding to heavy and light chains (70 and 25 kDa, respectively) and a Fab-fragment (50 kDa), no distinct bands were visible with bacterial lysate (eluted proteins from 34 × 10^3 cells) Only a weak band with a size of approximately 60 kDa was observed. Protein bands are marked with arrows in Fig. 4E. This molecular mass corresponds to protein A. When the proteins were directly extracted from the separated cells and the cultivation step was eliminated, protein bands in 16 × 10^3 cells were observable. After 3 h of enrichment cultivation, it was possible to extract Zn-proteins from the first 100 cells in an amount detectable with silver staining (tens ng per lane). Results are shown in Fig. 4C. Proteins of molecular masses of 70, 60, 45, 30, 25, and less than 10 kDa were present in the eluate. The source of proteins was either from bacterial (Zn-proteins or their ligands) or human IgG used for bacterial immunoseparation, which originated from bacterial surface Zn-proteins [85]. Total Zn(II) content was determined in the eluted proteins, initial bacterial lysate, and retented proteins. Zn(II) was present in concentrations from 0.023 to 2.22 µg/mL with concentrations dependent on an exponential trend. If Zn(II) contents in lysate, retentate, and eluate are considered,

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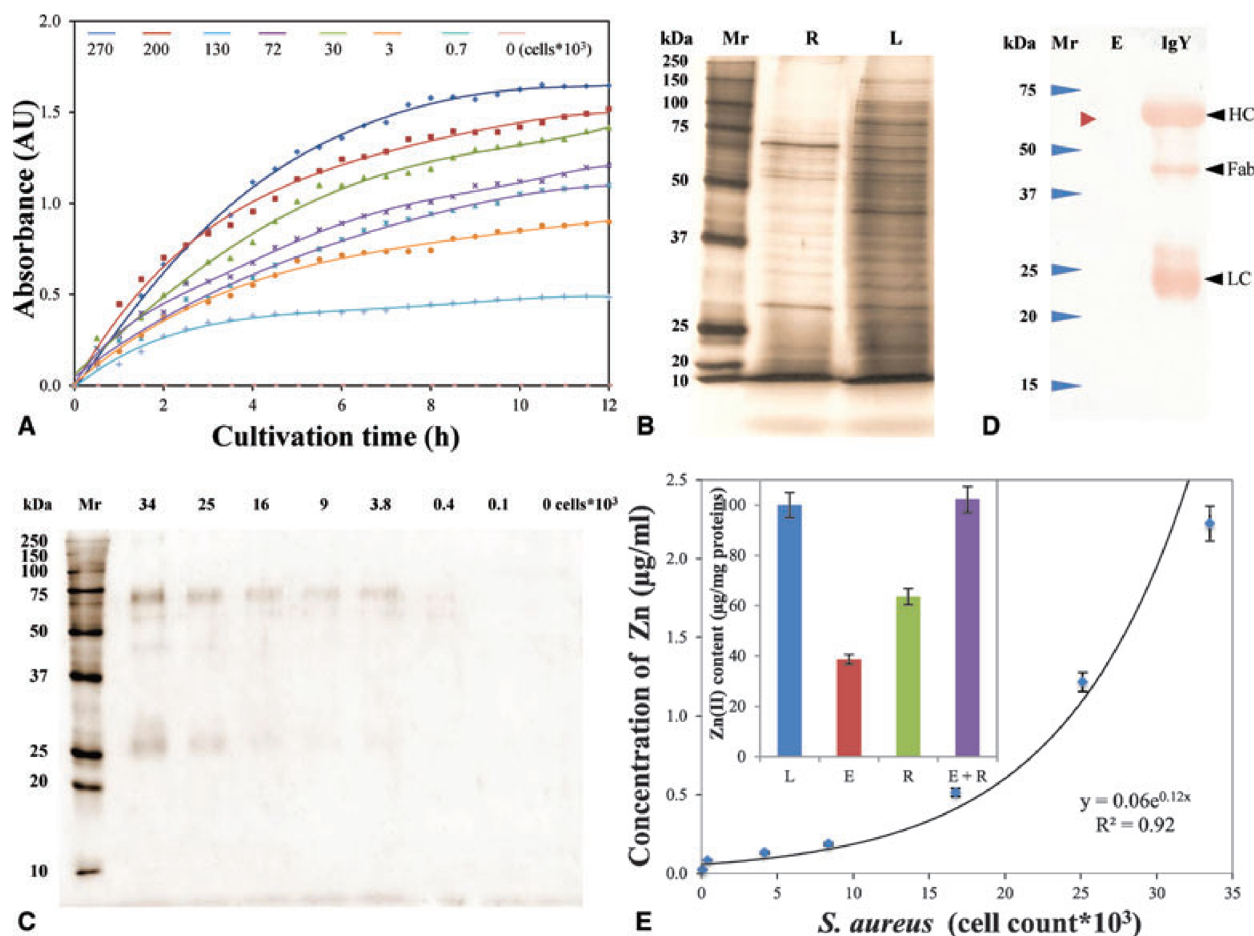

Figure 4. (A) Growth of the immunoextracted bacteria (0–270.10^3 cells in original culture); (B) SDS-PAGE of the bacterial lysate before and after immunoextraction. (C) SDS-PAGE of proteins eluted from beads modified with chicken anti-Zn antibody, R–bacterial lysate after immunoextraction (retentate), L–bacterial lysate, 300 ng of total proteins in 10 µL of lysate and 10 µL of retentate was loaded. (D) Western blot of the eluted proteins (200 ng) and 50 ng of chicken anti-Zn antibody (Ab) with secondary rabbit anti-chicken/HRP conjugate to verify the identity of eluted proteins. (E) Zn(II) concentration in eluted proteins, in inset: Zn(II) content in bacterial lysate, elutate, and retentate.

an initial value of 102% for lysate was calculated (inset in Fig. 4E).

4 Concluding remarks

The emergence of drug-resistant bacteria and new or changing infectious pathogens is an important public health problem. Transmission of these pathogens in an acute care setting may occur frequently if proper precautions are not taken [86]. Despite several guidelines and an abundance of literature on the prevention of transmission of epidemiologically important organisms in the healthcare setting, there are still needs to have an easy-to-use and rapid method for isolation of bacteria. The optimized assay was successfully applied on analysis of real samples. Based on the results obtained, we are able to analyze 48 real samples within 6 h. Besides the presence of bacteria, their zinc proteome can be investigated.

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