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PAPER

Use of magnetic hydrazide-modified polymer microspheres for enrichment of *Francisella tularensis* glycoproteins

Daniel Horák,^{*a} Lucie Balonová,^b Benjamin F. Mann,^c Zdeněk Plichta,^a Lenka Hernychová,^b Milos V. Novotny^c and Jiří Stulík^b

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The field of microbial proteomics has currently experienced a boom in the discovery of glycosylated proteins of various pathogenic bacteria as potential mediators of host–pathogen interactions. The presence of glycoproteins has recently been discovered in a Gram-negative pathogenic bacterium *Francisella tularensis*, utilizing glycoprotein detection and isolation techniques in combination with mass spectrometry. The isolation of glycoproteins is a prerequisite for their subsequent mass-spectrometric identification. Current glycoprotein isolation/enrichment methods comprise lectin affinity chromatography, aminophenylboronic acid and hydrazide-based enrichment. The use of magnetic microspheres containing functional groups is nowadays among state-of-art separation methodologies owing to an ease of manipulation, a speed of separation, and a minimum of non-specific protein adsorption. In the present study, novel magnetic hydrazide-modified poly(2-hydroxyethyl methacrylate) (PHEMA) microspheres were developed using a multi-step swelling and polymerization method with subsequent precipitation of magnetic iron oxides within the pores of the particles. The microspheres had a regular shape, size of 4 μm and contained 0.18 mmol hydrazide groups per g; the magnetic microspheres were employed for specific enrichment of *Francisella tularensis* glycoproteins. Effectiveness of the newly prepared magnetic microspheres for glycoprotein enrichment was proved by comparison with commercial hydrazide-functionalized microparticles.

Introduction

Glycosylation is one of the most ubiquitous post-translational modifications of proteins occurring in all domains of life—eukarya, bacteria, and archaea.^{1–3} It has been documented that glycosylation has an influence on the protein properties and plays a crucial role in many biological processes such as cell-to-cell recognition, cell adhesion and modulating the immune response.^{4–6} In some bacteria, glycosylation has been found to influence virulence-associated properties of the microbes, which has led to the decreased ability to colonize the host organisms.^{7,8} Protein glycosylation thus appears to contribute to virulence of particular bacteria.

Francisella tularensis (*F. tularensis*) is a Gram-negative, facultative intracellular bacterium that is pathogenic for many mammalian species, including humans. This highly infectious

bacterium is the causative agent of tularemia, the disease, for which no licensed vaccine is currently in hand.⁹ Recently, we suggested a potential role of a protein glycosylation in the virulence of *F. tularensis* and concordantly conducted an investigation of the *F. tularensis* glycoproteome. Several dozen putative *F. tularensis* glycoproteins were thus identified through the bottom-up proteomics approach, in which a saccharide-specific detection on the basis of both hydrazide and lectin enrichment techniques was applied in combination with mass spectrometry.¹⁰ The achievement of the glycoprotein enrichment using lectins is however fairly dependent on the lectin specificity toward a particular glycan structure present in the glycoprotein. The use of isolation/enrichment technique based on chemical reaction between the glycan moiety and a certain functional group immobilized on a solid carrier can overcome this limitation as it allows for isolation of the entire glycoprotein pool, regardless of the glycan structure. Recently, the use of hydrazide-modified solid support for isolation, identification and quantification of *N*-linked glycopeptides from human plasma and sera has been reported.¹¹ In the study, oxidized glycoproteins were reacted with immobilized hydrazide under mildly acidic conditions to form stable hydrazone linkages. Generally, *cis*-diol groups of glycans are oxidized to form aldehydes followed by their covalent attachment to a hydrazide-modified solid support.

^aInstitute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Heyrovský Sq. 2, 162 06 Prague, Czech Republic. E-mail: horak@imc.cas.cz

^bInstitute of Molecular Pathology, Faculty of Military Health Sciences, University of Defence, Trebešská 1575, 500 01 Hradec Králové, Czech Republic

^cDepartment of Chemistry, Indiana University, 800 E Kirkwood Avenue, 47405 Bloomington, IN, USA

Nonglycosylated proteins are removed by washing, while captured glycoproteins are proteolytically digested. Nonglycosylated peptides are released whereas glycopeptides remain covalently immobilized on the carrier. While eukaryotic glycopeptides can be released from the carrier through enzymatic digestion with PNGase F and analyzed,¹¹ bacterial glycans are resistant to this peptide-*N*-glycanase. Therefore, the information on the bacterial glycopeptides, including the composition of the attached glycans, is not acquired. Nevertheless, the identification of captured glycoproteins can be achieved indirectly through the analysis of nonglycosylated peptides originated from captured glycoproteins. Such an indirect approach of glycoprotein identification in *F. tularensis* was performed in the present study, utilizing the newly synthesized magnetic hydrazide-modified poly(2-hydroxyethyl methacrylate) (PHEMA) microspheres.

PHEMA was intentionally selected as a matrix of the microspheres since it is a hydrophilic, biocompatible, biologically inert polymer known for reduced non-specific protein adsorption and cell adhesion.¹² PHEMA properties can be tailored for the specific application. 2-Hydroxyethyl methacrylate (HEMA) easily undergoes copolymerization with a plethora of monomers making thus introduction of functional groups (carboxyl, amino, sulfhydryl, *etc.*) possible. The functional groups are then available for attachment of target biomolecules. Moreover, a great advantage of magnetic carriers is the ease with which they can be separated from solutions or complex mixtures by exposure to a magnetic field. Conventional separations using centrifugation or filtration can be thus avoided. Biomedical applications of magnetic particles have been recently reviewed.^{13–17} In this report, the newly developed magnetic hydrazide-modified PHEMA microspheres were compared with commercial SiMAG-Hydrazide microspheres¹⁸ in terms of enrichment of *F. tularensis* glycoproteins.

Experimental

Materials

Styrene (Kaučuk Kralupy, Czech Republic) and ethylene dimethacrylate (EDMA; Röhm; Darmstadt, Germany) were vacuum distilled. 2-(Methacryloyl)oxyethyl acetate (HEMA-Ac) was obtained from HEMA (Röhm) and acetic anhydride and 2-[(methoxycarbonyl)methoxy]ethyl methacrylate (MCMEMA) were prepared according to the earlier described procedure.¹⁹ Cyclohexyl acetate was obtained from cyclohexanol and acetic anhydride. FeCl₂·4H₂O, 2-hydroxyethyl cellulose, (hydroxypropyl)methyl cellulose (Methocel 90 HG), sodium dodecyl sulfate (SDS), Tween 20, dibutyl phthalate (DBP) and benzoyl peroxide (BPO) were products of Fluka (Buchs, Switzerland), sodium persulfate was from Lachema (Brno, Czech Republic), and hydrazine hydrate was from Xenon (Lodz, Poland). SiMAG-Hydrazide microspheres (1 μm particle size) were from Chemicell (Berlin, Germany). RapiGest surfactant was purchased from Waters (Milford, USA), Affi-Gel Hz coupling buffer was from Biorad (Hercules, USA), and protease inhibitor cocktail mini-EDTA free was obtained from Roche (Mannheim, Germany). If not specified, all other chemicals were obtained from Sigma-Aldrich (St. Louis, USA). A chemically defined complete Chamberlain medium was prepared according to the method of Chamberlain.²⁰

Preparation of magnetic hydrazide-PHEMA microspheres

Preparation of monodisperse macroporous P(HEMA-Ac-co-MCMEMA-co-EDMA) microspheres. First, polystyrene (PS) seeds were obtained by the sodium persulfate-initiated emulsifier-free emulsion polymerization of styrene by modification of the earlier published procedure.²¹ Polystyrene latex (containing 0.3 g PS) was then activated by repeated swelling in an emulsion of DBP (4.3 g) in 0.25% SDS solution (17.5 ml) under sonication (4710 Ultrasonic Homogenizer; Cole-Parmer Instruments, Chicago, USA). A mixture of BPO (30 mg), 2-(methacryloyl)oxyethyl acetate (HEMA-Ac; 1.5 g; 8.7 mmol), 2-[(methoxycarbonyl)methoxy]ethyl methacrylate (MCMEMA; 0.3 g; 1.48 mmol) and EDMA (1.2 g) in 0.1% SDS solution (7.5 ml) was shortly sonicated and DBP-swollen PS latex (2 ml) was swollen in the mixture for 16 h under mild stirring using a SB3 rotator (30 rpm; Barloworld Scientific; Stove, UK) and the mixture was transferred into a 40 ml reaction vessel equipped with an anchor-type stirrer. Separately, an emulsion of cyclohexyl acetate (4 g) in 0.1% SDS solution (10 ml) was obtained by sonication for 3 min and added to the monomer-swollen PS particles; the mixture was stirred (300 rpm) for 1 h under CO₂ atmosphere. 2 wt% 2-hydroxyethyl cellulose aqueous solution (2 ml), 2 wt% Methocel 90 HG aqueous solution (2 ml) and citric acid (30 mg in 0.5 ml water) were added and the mixture was stirred (600 rpm) under CO₂ atmosphere. Polymerization was started by heating at 70 °C for 16 h. The resulting macroporous P(HEMA-Ac-co-MCMEMA-co-EDMA) microspheres were separated by filtration, washed with water, 0.05% Tween 20, ethanol, and water and filtered *via* a sieve (32 μm mesh size). If P(HEMA-Ac-co-MCMEMA-co-EDMA) microspheres were hydrolyzed, they were treated with 0.5 N NaOH at 60 °C for 16 h and washed with water.

Transformation to hydrazide-PHEMA microspheres. Macroporous P(HEMA-Ac-co-MCMEMA-co-EDMA) microspheres (1.5 g) were dispersed in 1,4-dioxane (20 ml) and 80% aqueous hydrazine solution (2 ml), the mixture sonicated, 0.05% Tween 20 (6 ml) added and the reaction proceeded at 22 °C for 72 h under mild shaking (50 rpm). Resulting microspheres (denoted as hydrazide-PHEMA) were washed with ethanol, water, 0.01 M HCl and water.

Precipitation of iron oxide in hydrazide-PHEMA microspheres. Hydrazide-PHEMA microspheres were dispersed in 17 wt% aqueous FeCl₂ solution (10 ml) under Ar, sonicated for 3 min, filtered, washed with the same solution and dried at 50 °C. The microspheres were then added in 0.4 M NH₄OH solution (40 ml), the mixture sonicated for 4 min and stirred (400 rpm) at 22 °C for 16 h in air. At the same time, hydrazide-PHEMA-Ac hydrolyzed to PHEMA. The microspheres were separated, 0.1 M HCl (1 ml) added to peptize the iron oxide outside of the microspheres, which was then removed by repeated washing with water.

Characterization

The microspheres were observed in a Quanta FEG 200F scanning electron microscope (SEM; FEI, Brno, Czech Republic) to determine number-average diameter (D_n), weight-average

diameter (D_w), and polydispersity index ($PDI = D_w/D_n$) characterizing width of the particle size distribution. The microspheres were also examined by a Paragon 1000 PC FTIR spectrometer (Perkin Elmer; Norwalk, USA) with a Specac MKII Golden Gate Single Reflection ATR System with diamond crystal and angle of ray incidence 45° . The iron content was analyzed by atomic absorption spectrometry (AAS Perkin-Elmer 3110) of an extract from a sample obtained by treatment with 70% perchloric and 65% nitric acid at 100°C for 30 min. Elemental analysis was performed on a Perkin-Elmer 2400 CHN apparatus.

Capture of glycoproteins by magnetic hydrazide-PHEMA microspheres

Bacterial strains and culture conditions. The *F. tularensis* subsp. *holarctica* strain FSC200 was kindly provided by the FOI Swedish Defense Research Agency (Dr Åke Forsberg, Umea, Sweden). Bacteria were grown, harvested, and lysed within a BioSafety Level 2 containment facility. Bacteria were cultured on McLeod agar supplemented with bovine hemoglobin and IsoVitaleX (Becton, Dickinson, Le Pont de Claix, France) at 36.8°C for 24–48 h. Colonies scraped from the plate were inoculated into Chamberlain medium and cultivated for 16 h at 36.8°C under constant shaking. The 16 h cultures were diluted with fresh Chamberlain medium ($OD_{600\text{nm}} = 0.1$) and grown until the late logarithmic growth phase of bacteria ($OD_{600\text{nm}} = 0.8$). Bacterial cells were collected by centrifugation at $9000g$ for 15 min at 4°C and the pellets were washed twice with cold PBS (pH 7.4). The resulting pellets were resuspended in PBS (pH 7.4). Protease inhibitor cocktail was added to a final dilution 1 : 50 (v/v).

Preparation of whole-cell lysates. The cells were disrupted using a French press twice at 110 MPa, while the resulting cell debris along with intact microbes were removed by centrifugation at $12\,600g$ for 30 min at 4°C . Benzonase nuclease ($361\text{ U } \mu\text{l}^{-1}$) was added to the supernatant, resulting in a final concentration of 150 U ml^{-1} of lysate.

Preparation of membrane protein-enriched fraction. Fractions enriched in the membrane proteins were collected by ultracentrifugation of whole-cell lysates at $115\,000g$ for 1 h at 4°C . The supernatant was discarded and the membrane pellet was resuspended in ice-cold PBS (pH 7.4), and then collected by centrifugation at $115\,000g$ for 30 min at 4°C . The final membrane protein-containing pellet was solubilized in a 10-fold diluted Affi-Gel Hz coupling buffer with addition of 0.2% RapiGest surfactant. Samples were then sonicated for 5 min in 5 s pulses with 1 s cooling periods after each pulse. Undissolved proteins were removed by centrifugation at $10\,000g$ for 5 min. Proteins were quantified by Bicinchoninic acid.

Glycoprotein capture on magnetic hydrazide-modified microspheres. The commercial SiMAG-Hydrazide and newly synthesized magnetic hydrazide-PHEMA microspheres were used for capturing glycoproteins. Briefly, proteins (1 mg) in a 10-fold diluted Affi-Gel Hz coupling buffer containing 0.2% RapiGest were oxidized in 10 mM sodium periodate for 1 h at room temperature in the dark under end-over-end rotation. The

unreacted sodium periodate was consequently removed by using a PD Mini Trap G-25 column (GE Healthcare; Uppsala, Sweden). The oxidized, desalted proteins were then added to the tested magnetic hydrazide-modified microspheres (2 mg) pre-equilibrated with coupling buffer. The coupling reaction was left overnight at room temperature under end-over-end rotation. The unbound non-glycoprotein fraction was removed using the magnetic separator. Next, the magnetic microspheres with captured glycoproteins were briefly washed with methanol (0.5 ml) and 8 M urea in 50 mM ammonium bicarbonate solution sequentially three times to remove nonspecifically adsorbed non-glycoproteins. The immobilized glycoproteins were denatured and reduced with 8 M urea in 50 mM ammonium bicarbonate solution containing 10 mM dithiothreitol for 1 h at 37°C , followed by the alkylation of protein cysteinyl residues using 20 mM iodoacetamide in 50 mM ammonium bicarbonate solution for 1.5 h at room temperature in the dark. After washing the microspheres with 50 mM ammonium bicarbonate, trypsin in a 50 mM ammonium bicarbonate was added (trypsin/protein = 1/100 w/w, based on the indirect calculation of the amount of the bound glycoproteins from the difference in protein concentration before and after coupling). The proteins were digested on the magnetic microspheres overnight at 37°C under end-over-end rotation. The trypsin-released non-glycopeptides were magnetically separated. The magnetic microspheres were washed with 80% acetonitrile (ACN), the eluent was added to the first collected fraction and vacuum-dried. The peptides were then reconstituted in a 0.1% trifluoroacetic acid (TFA) containing 5% of ACN and desalted on C18 ZipTip pipette tips (Millipore, Bedford, USA) pre-equilibrated sequentially with methanol, 0.1% TFA in 80% ACN, and 0.1% TFA in 5% ACN. Desalted peptides were vacuum-dried and reconstituted in 0.1% formic acid (FA) in 5% ACN for subsequent LC/ESI-MS/MS analysis.

LC/ESI-MS/MS analysis and data processing. An aliquot of protein digest corresponding to $1\text{ }\mu\text{g}$ of total proteins was analyzed by C18 nanoscale reversed-phase liquid chromatography coupled on-line to an LTQ-FT-ICR mass spectrometer (Thermo Finnigan; San Jose, USA). Digested samples were pre-concentrated on a micro-precolumn C18 cartridge ($300\text{ }\mu\text{m i.d.} \times 5\text{ mm}$) (LC Packings; Sunnyvale, USA). After loading and washing the peptides for 10 min with mobile phase A (water/ACN/FA = 97/3/0.1), the trapping column was switched in-line with the analytical column. The separation of peptides was conducted with a C18 column ($75\text{ }\mu\text{m i.d.} \times 150\text{ mm}$) packed in-house with Magic C18AQ particles ($200\text{ }\text{Å}$, $3\text{ }\mu\text{m}$) from Michrom Bioresources (Auburn, CA, USA) with a linear gradient, from 3 to 55% phase B (water/ACN/FA = 3/97/0.1) over a period of 45 min and ramped from 55 to 80% ACN over 10 min. The column eluent was electrosprayed into the mass spectrometer using 1.5 kV spraying voltage. The FT-MS spectra were acquired in the mass range from 300 to 2000 m/z followed by the fragmentation of the five most intense peaks. Collision activation was performed using helium gas at a normalized collision energy of 35%. Acquisition of data was controlled by Xcalibur software (Thermo Finnigan). Acquired RAW data were converted into MGF files using Turbo RAW2MGF converter v.1.0.7 and then subjected to MASCOT searching against the *F. tularensis* subsp. *holarctica* OSU18 database. The searching criteria were set as

follows: trypsin was used as the protease, up to 1 missed cleavage was allowed, 1+, 2+ and 3+ ions, carbamidomethylation of cysteine as a fixed modification and oxidation of methionine as a variable modification. Data were then filtered with ProteinParser v.2.1 software²² to reject peptides with a MOWSE probability score threshold less than 20 and peptides containing KK, KR, KR or RR motifs. Only peptides containing more than six amino acids and a mass greater than 600 Da were accepted.

Bioinformatic analysis. The following bioinformatic tools were used to categorize the identified potential glycoproteins: (i) the PSORTb v.2.0. program to predict protein localization²³ and (ii) the LipoP program to identify lipoproteins and signal peptidase I-cleavable proteins.²⁴

Results and discussion

Macroporous P(HEMA-Ac-co-MCMEMA-co-EDMA) microspheres

The multi-step swelling and polymerization method pioneered by Ugelstad *et al.*²⁵ was chosen for preparation of particles in this report as it provides non-aggregating monodisperse polymer microspheres. The method is based on monodisperse PS seeds which are prepared by emulsion-free emulsion polymerization.²¹ The seeds were 0.69 μm in size and monodispersity was documented by low polydispersity index $\text{PDI} = 1.004$. The PS seeds were pre-swelled (activated) by incorporating a substantial amount of DBP in the particles prior to swelling with monomers and subsequent polymerization. As a principal monomer, HEMA-Ac was used for swelling since it is insoluble in water (contrary to HEMA) in which subsequent polymerization proceeds. This is a big advantage since undesirable emulsion and solution polymerization (resulting in tiny polymer particles) are thus avoided. In addition to HEMA-Ac, MCMEMA (0.5 mmol g^{-1} of particles) was incorporated in the microspheres to introduce reactive methyl ester groups to facilitate subsequent modification with hydrazine *via* hydrazide bond formation.

Monomer-swollen PS seeds were finally suspension polymerized in water using 2-hydroxyethyl cellulose and (hydroxypropyl)-methyl cellulose as steric stabilizers to prevent particle aggregation. BPO was the preferred initiator for polymerization since it is insoluble in water. To confirm the presence of carboxyl groups, documenting thus the incorporation of MCMEMA in the PHEMA-Ac microspheres, ATR-FTIR spectra of P(HEMA-Ac-co-MCMEMA-co-EDMA) microspheres before and after hydrolysis were recorded (Fig. 1). In the latter microspheres, a peak at 1605 cm^{-1} ascribed to carboxyl groups suggests that the hydrolysis of methyl ester of P(HEMA-Ac-co-MCMEMA-co-EDMA) with NaOH was successful.

In order to make prospective precipitation of iron oxide inside the microspheres possible, they have to contain micropores. As it is well known that the formation of porous structure in the microspheres requires the presence of sufficient amounts of both crosslinking agent and porogen in the reaction mixture,²⁶ EDMA crosslinker (40 wt% relative to all monomers) and cyclohexyl

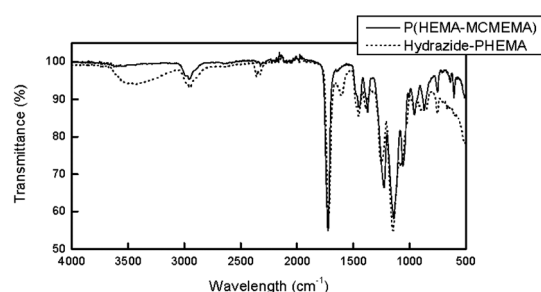


Fig. 1 ATR-FTIR spectra of P(HEMA-Ac-co-MCMEMA-co-EDMA) microspheres (—) before and (···) after hydrolysis with 0.5 N NaOH.

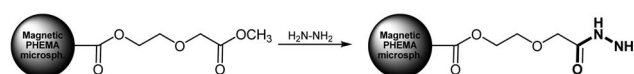
acetate porogen (60 wt% relative to organic phase) were incorporated in the swelling mixture. After completion of the polymerization, porogen was easily removed by washing, thereby leaving pores within the microsphere structure. At the same time, crosslinking with EDMA prevented dissolution of the microspheres in the reaction medium.

Hydrazide-PHEMA microspheres

In this report, in order to obtain hydrazide-PHEMA microspheres, reaction of P(HEMA-Ac-co-MCMEMA-co-EDMA) with hydrazine was carried out in 1,4-dioxane (Scheme 1). The presence of hydrazide groups in hydrazide-PHEMA microspheres was confirmed by comparing the results of elemental analysis of neat (non-hydrazided) and hydrazide-PHEMA microspheres; the nitrogen content in the latter microspheres was increased (0.36 mmol N g^{-1} ; Table 1). It can be thus estimated that 0.18 mmol hydrazide groups were available per g of the microspheres. According to SEM, the hydrazide-PHEMA microspheres had a regular spherical shape, were 4 μm in diameter and narrow size distribution was characterized by $\text{PDI} = 1.01$ (Fig. 2a).

Magnetic hydrazide-PHEMA microspheres

In the next step, magnetic hydrazide-PHEMA microspheres were prepared. To avoid the danger of reaction between FeCl_3 and hydrazide, magnetic iron oxides were formed *in situ* inside the pores of the hydrazide-PHEMA particles from neat ferrous chloride and ammonium hydroxide, a distinct difference from earlier methodologies that reported the use of a mixture of ferrous and ferric chlorides.^{27,28} The reaction consists of several steps. First, $\text{Fe}(\text{OH})_2$ is formed and oxidized by atmospheric oxygen to $\text{Fe}(\text{OH})_3$, which, in combination with $\text{Fe}(\text{OH})_2$, immediately results in Fe_3O_4 . The magnetic hydrazide-PHEMA microspheres contained 13.1 wt% Fe as determined by AAS. At the same time, ammonium hydroxide induced hydrolysis of

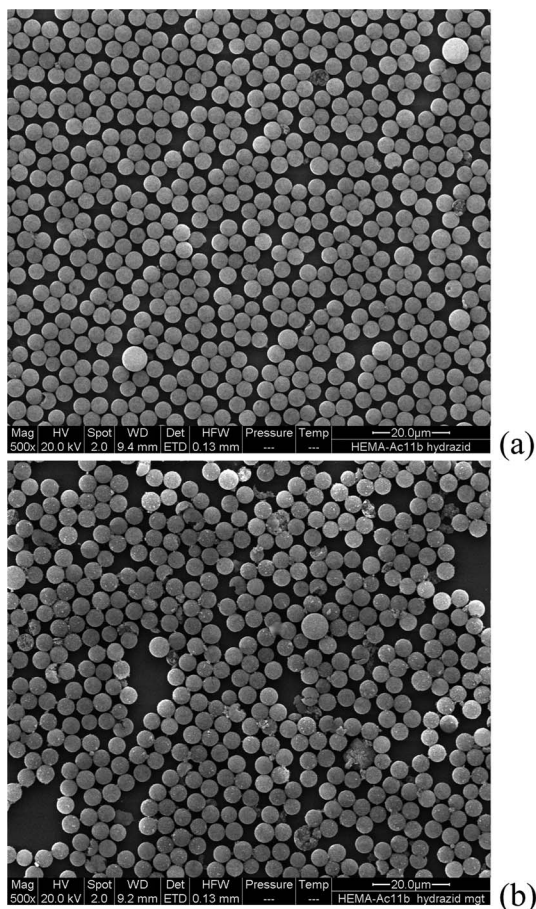


Scheme 1 Hydrazidation of P(HEMA-Ac-co-MCMEMA-co-EDMA) microspheres.

Table 1 Results of elemental analysis^a

Microspheres	C (wt%)	H (wt%)	N (wt%)
P(HEMA-Ac-co-MCMEMA-co-EDMA)	57.42	6.77	0.03
Hydrazide-PHEMA	57.05	6.87	0.50
Magnetic hydrazide-PHEMA	47.34	5.40	0.45

^a Relative error was 0.1.

**Fig. 2** SEM of (a) hydrazide-PHEMA microspheres and (b) magnetic hydrazide-PHEMA microspheres (13.1 wt% Fe).

2-acetoxyethyl units in the P(HEMA-Ac-co-MCMEMA-co-EDMA) microspheres to free 2-hydroxyethyl groups of PHEMA. The hydrazide-PHEMA particle size and size distribution were not changed during the formation of iron oxides as documented by the SEM micrograph (Fig. 2b).

Glycoprotein capture on magnetic hydrazide-modified microspheres

The newly developed 4 μm magnetic hydrazide-PHEMA microspheres were compared with commercial 1 μm SiMAG-Hydrazide microspheres for capturing *F. tularensis* glycoproteins. The capture is based on the irreversible reaction of hydrazide groups of the microspheres with aldehydes generated by oxidation of *cis*-diol groups of glycoprotein's glycans

(Scheme 2). A total of three biological replicates of the membrane protein-enriched fraction from *F. tularensis* were incubated with both types of magnetic hydrazide microspheres and the nonglycosylated peptide mixture that originated from captured glycoproteins was analyzed using on-line nanoLC-ESI-MS/MS mass spectrometry. Putative glycoproteins were identified by comparing experimental data with theoretical masses from the *F. tularensis* subsp. *holarctica* OSU18 protein sequences database (NCBI Reference sequence: NC_008369.1). Only the proteins that were found in all three experiments were further considered, giving the total number of 128 and 133 proteins for magnetic hydrazide-PHEMA and SiMAG-Hydrazide microspheres, respectively. The identified proteins are listed in Tables 2–4.

The localization of the identified proteins within the bacterial cell was determined using the PSORTb program. However, PSORTb is not able to determine lipoproteins, which comprise an important class among the membrane proteins. For this reason, the LipoP lipoprotein prediction program was additionally applied to identify proteins with signal peptidase I or II (lipoprotein) cleavage site. These proteins are predicted to pass through the inner membrane of the cell wall of Gram-negative bacteria and are therefore the protein subset that may be glycosylated. Proteins without a signal peptide (non-secretory proteins) are less likely to be glycosylated owing to the biosynthetic pathway of glycoproteins which occurs after crossing the inner membrane.²⁹ The cytosolic glycosylation, however, cannot be entirely excluded from consideration.³⁰

The identified proteins were compared with those identified in our previous glycoproteomics studies to demonstrate a specificity of a hydrazide group to glycosylation. As is obvious from Table 4, 64 of 128 and 64 of 133 proteins herein identified using magnetic hydrazide-PHEMA and SiMAG-Hydrazide microspheres, respectively, were already described as potentially glycosylated using Pro-Q Emerald carbohydrate-specific staining, DIG Glycan Differentiation kit and/or lectin affinity chromatography with various lectins.¹⁰ A total of 43 proteins not previously identified as glycosylated were common to both hydrazide-modified microspheres. Additionally, 25 previously not identified proteins were captured only on SiMAG-Hydrazide and 20 previously not detected proteins were isolated only with newly synthesized magnetic hydrazide-PHEMA microspheres.

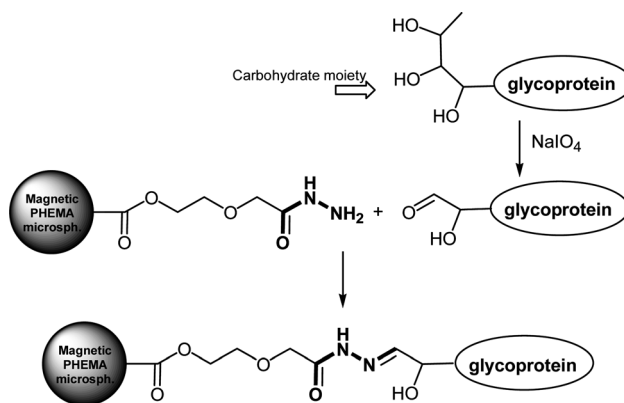
**Scheme 2** Isolation of glycoproteins on magnetic hydrazide-modified microspheres.

Table 2 List of *F. tularensis* proteins isolated on magnetic SiMAG-Hydrazide microspheres

Gene locus ^a	Protein name	(M_w^b /kDa)/pI	PSORTb ^c	LipoP ^d
FTH_0010	Sulfur transferase	15.89/9.22	?	cyt
FTH_0038	Probable multidrug efflux pump	37.24/9.17	CM	TMH
FTH_0039	Putative uncharacterized protein	15.30/5.16	?	SPII
FTH_0066	30S ribosomal protein S20	10.15/10.96	cyt	cyt
FTH_0067	Elongation factor 4	65.57/5.42	CM	cyt
FTH_0108	Conserved protein PdpC	156.00/9.17	cyt	cyt
FTH_0117	Conserved protein (conserved protein PdpB)	127.47/9.63	OM	cyt
FTH_0124	FeoB family ferrous iron (Fe ²⁺) uptake protein	81.46/8.55	CM	cyt
FTH_0139	ABC superfamily ATP binding cassette transporter, ABC protein	49.45/4.72	CM	cyt
FTH_0159	Universal stress protein	30.4/5.78	cyt	cyt
FTH_0169	50S ribosomal protein L34	5.18/12.96	cyt	cyt
FTH_0184	Cytochrome d ubiquinol oxidase subunit I	64.28/7.63	CM	TMH
FTH_0186	Cytochrome o ubiquinol oxidase subunit II	34.46/7.02	CM	TMH
FTH_0187	Cytochrome o ubiquinol oxidase subunit I	76.20/8.58	CM	TMH
FTH_0189	Cytochrome o ubiquinol oxidase subunit IV	12.19/6.37	CM	TMH
FTH_0219	Ribosomal protein S2	26.42/8.94	cyt	cyt
FTH_0228	Ribosomal protein S7	17.82/10.82	cyt	cyt
FTH_0230	Ribosomal protein S10	11.90/9.79	cyt	cyt
FTH_0231	Ribosomal protein L3	22.31/9.65	cyt	cyt
FTH_0232	50S ribosomal protein L4	22.55/10.29	cyt	cyt
FTH_0234	Ribosomal protein L2	30.40/11.54	cyt	cyt
FTH_0237	Ribosomal protein S3	24.88/10.10	cyt	cyt
FTH_0238	Ribosomal protein L16	15.71/11.43	cyt	cyt
FTH_0239	Ribosomal protein L29	7.80/10.34	cyt	cyt
FTH_0241	Ribosomal protein L14	13.23/10.51	cyt	cyt
FTH_0242	50S ribosomal protein L24	11.48/9.73	cyt	cyt
FTH_0243	Ribosomal protein L5	20.00/9.75	cyt	cyt
FTH_0246	Ribosomal protein L6	19.08/9.85	cyt	SPI
FTH_0248	Ribosomal protein S5	17.56/9.99	cyt	cyt
FTH_0250	Ribosomal protein L15	15.10/10.24	cyt	cyt
FTH_0251	Preprotein translocase subunit secY	48.46/10.01	CM	TMH
FTH_0253	Ribosomal protein S13	13.38/11.54	cyt	cyt
FTH_0254	Ribosomal protein S11	13.76/10.85	cyt	cyt
FTH_0255	Ribosomal protein S4	23.26/9.85	cyt	cyt
FTH_0257	Ribosomal protein L17	16.78/10.72	cyt	cyt
FTH_0268	Glutamate dehydrogenase (NADP(+))	49.16/6.49	?, MLS	cyt
FTH_0295	Acetyl-CoA carboxylase α subunit	35.44/8.07	cyt	cyt
FTH_0310	Pyruvate dehydrogenase (acetyl-transferring)	100.27/5.65	cyt	cyt
FTH_0334	Probable OmpA family protein	23.29/4.95	OM	SPII
FTH_0357	LemA family protein	21.97/5.63	cyt	cyt
FTH_0384	Type IV pili fiber protein (PilA)	13.59/9.06	?, MLS, fimbrial	SPI
FTH_0384	Type IV pili fiber protein (PilA)	13.59/9.06	?, MLS, fimbrial	SPI
FTH_0403	Putative uncharacterized protein	32.50/8.46	cyt	cyt
FTH_0447	Phosphatidylserine decarboxylase	32.13/9.72	CM	cyt
FTH_0462	Possible neuraminidase	42.6/9.52	CM	TMH
FTH_0463	Soluble lytic murein transglycosylase	76.93/9.09	PP	SPI
FTH_0515	Cell division topological specificity factor protein MinE	10.18/8.03	cyt	cyt
FTH_0516	Septum site-determining protein MinD	30.8/6.85	cyt, MLS	cyt
FTH_0519	Ribosomal protein L28	8.94/11.06	cyt	cyt
FTH_0535	DNA topoisomerase (ATP-hydrolyzing)	97.13/5.41	cyt	cyt
FTH_0539	UDP-3-O-[3-hydroxymyristoyl] glucosamine <i>N</i> -acyltransferase	35.8/5.80	cyt	cyt
FTH_0540	(3 <i>R</i>)-Hydroxymyristoyl-[acyl-carrier-protein] dehydratase	18.15/6.83	cyt	cyt
FTH_0541	Acyl-[acyl-carrier-protein]-UDP- <i>N</i> -acetylglucosamine <i>O</i> -acyltransferase	28.13/8.37	cyt	cyt
FTH_0542	1,4- α -Glucan branching enzyme	43.09/9.34	cyt	cyt
FTH_0554	Signal peptidase I	32.87/8.82	CM	TMH
FTH_0585	Acyl-CoA dehydrogenase	84.19/8.45	?	cyt
FTH_0586	Long-chain-fatty-acid-CoA ligase	63.07/7.57	CM	cyt
FTH_0589	Putative uncharacterized protein	33.26/9.53	CM	TMH
FTH_0592	dTDP-glucose 4,6-dehydratase	65.71/8.17	CM	TMH
FTH_0593	Galactosyltransferase	23.78/10.21	CM	SPI
FTH_0594	UDP-glucose 4-epimerase	29.96/9.46	?	SPI
FTH_0595	Galacturonosyltransferase	41.60/9.39	cyt	cyt
FTH_0600	Asparagine synthase (glutamine-hydrolyzing)	71.81/7.91	cyt	cyt
FTH_0604	Glycosyltransferase	32.98/6.51	cyt	cyt
FTH_0628	Possible glycosyltransferase	35.58/7.16	CM	cyt
FTH_0659	Conserved hypothetical protein	4.51/8.90	cyt	TMH
FTH_0689	Probable multidrug resistance efflux pump	37.83/9.36	?	cyt
FTH_0719	Ribonuclease E	95.95/7.12	cyt	cyt
FTH_0799	Bifunctional 1-pyrroline-5-carboxylate dehydrogenase/proline dehydrogenase	150.39/7.97	cyt	cyt
FTH_0800	APC family amino acid-polyamine-organocation transporter	55.00/9.27	CM	TMH
FTH_0836	Preprotein translocase subunit	12.87/10.55	CM	SPI

Table 2 (Contd.)

Gene locus ^a	Protein name	(M _w ^b /kDa)/pI	PSORTb ^c	LipoP ^d
FTH_0837	RND superfamily resistance-nodulation-cell division antiporter	69.67/9.52	CM	TMH/SPI
FTH_0886	Probable membrane protease subunit HflK	40.42/8.97	cyt	cyt
FTH_0887	Membrane protease subunit HflC	34.59/9.97	?	cyt
FTH_0928	50S ribosomal protein L25	10.90/9.26	cyt	cyt
FTH_1002	Ribosomal protein L9	16.45/5.55	cyt	cyt
FTH_1026	Ribosomal protein S21	7.84/11.4	cyt	cyt
FTH_1071	Thioredoxin family protein	39.69/4.89	?	SPII
FTH_1078	Putative uncharacterized protein	16.87/7.40	?	TMH
FTH_1107	Inositol-phosphate phosphatase	28.87/8.87	cyt	cyt
FTH_1112	3-Oxoacyl-[acyl-carrier-protein] synthase	44.3/5.62	cyt	cyt
FTH_1117	Fatty acid/phospholipid synthesis protein PlsX	37.84/9.62	cyt	cyt
FTH_1162	Ribosomal protein S9	10.09/10.35	cyt	cyt
FTH_1163	Ribosomal protein L13	15.94/10.03	cyt	cyt
FTH_1197	Aminotransferase	47.07/9.06	cyt	cyt
FTH_1216	Sec family Type II general secretory pathway signal recognition particle protein Ffh	50.31/10.31	CM	cyt
FTH_1267	Oxidoreductase	30.80/9.33	cyt	cyt
FTH_1281	Acetyl-CoA carboxylase β subunit	33.65/8.96	cyt	cyt
FTH_1354	ATP-dependent RNA helicase	64.01/8.91	cyt	cyt
FTH_1366	Ribosomal protein L20	13.34/10.81	cyt	cyt
FTH_1377	Putative uncharacterized protein	44.26/9.77	?	TMH
FTH_1379	Probable capsule biosynthesis protein	44.90/7.41	CM	TMH
FTH_1415	Ribosomal protein L21	11.56/10.04	cyt	cyt
FTH_1418	Sec family Type II general secretory pathway preprotein translocase SecA subunit	103.56/5.29	cyt, MLS	cyt
FTH_1424	M41 family endopeptidase FtsH/HflB	70.74/5.42	CM	TMH
FTH_1443	Bifunctional FAD/FMN dehydrogenase/Fe-S oxidoreductase	114.57/8.64	cyt	cyt
FTH_1487	Polyribonucleotide nucleotidyltransferase	75.53/5.68	cyt	cyt
FTH_1488	Ribosomal protein S15	10.36/10.13	cyt	cyt
FTH_1489	Peptidoglycan glycosyltransferase	62.73/8.77	CM	TMH
FTH_1492	Probable acyl-coenzyme A synthetase	78.58/9.04	CM	cyt
FTH_1503	Conserved hypothetical protein	69.85/10.14	CM	TMH
FTH_1536	Conserved hypothetical protein	25.35/6.96	?	cyt
FTH_1537	Acetyl-CoA carboxylase biotin carboxylase subunit	50.05/6.85	cyt	cyt
FTH_1551	S49 family SohB endopeptidase	38.07/9.25	CM	TMH
FTH_1558	Glycosyltransferase, group 2 family protein	36.08/8.69	CM	cyt
FTH_1599	Conserved hypothetical protein	49.32/4.33	?, MLS	SPI
FTH_1609	ABC superfamily ATP binding cassette transporter, ATP-binding protein	66.68/8.69	CM	SPI
FTH_1612	RND family efflux transporter, MFP subunit	50.09/10.12	CM	cyt
FTH_1613	RND superfamily resistance-nodulation-cell division:proton (H ⁺) antiporter	112.50/5.40	CM	TMH
FTH_1617	Conserved hypothetical protein	38.46/9.50	CM	SPI
FTH_1642	Cell division protein	92.02/5.63	CM	TMH
FTH_1674	Ribosomal protein L19	13.31/10.53	cyt	cyt
FTH_1677	Ribosomal protein S16	9.07/10.61	cyt	cyt
FTH_1682	DNA-directed RNA polymerase subunit β	157.39/5.97	cyt	cyt
FTH_1687	Ribosomal protein L1	24.63/9.50	cyt	cyt
FTH_1691	Elongation factor Tu	43.39/4.87	cyt	cyt
FTH_1696	Glycerol-3-phosphate dehydrogenase	57.78/8.36	?, MLS	cyt
FTH_1708	Aconitate hydratase	102.70/5.30	cyt	cyt
FTH_1719	Dihydrolipoyllysine-residue succinyltransferase	52.75/4.89	cyt	cyt
FTH_1720	Oxoglutarate dehydrogenase (succinyl-transferring)	105.67/6.01	cyt	cyt
FTH_1721	Succinate dehydrogenase	27.2/8.42	CM	cyt
FTH_1722	Succinate dehydrogenase	65.86/6.14	CM	cyt
FTH_1726	MFS family major facilitator transporter	47.16/9.78	CM	TMH
FTH_1732	H(+)-transporting two-sector ATPase	49.87/4.89	cyt	cyt
FTH_1734	H(+)-transporting two-sector ATPase	55.54/4.68	cyt	cyt
FTH_1736	H(+)-transporting two-sector ATPase	17.38/7.54	CM	cyt
FTH_1738	ATP synthase subunit a (ATP synthase F0 sector subunit a)	30.00/6.22	CM	cyt
FTH_1760	NADH dehydrogenase (ubiquinone)	87.36/5.09	cyt, MLS	cyt
FTH_1761	NADH dehydrogenase (ubiquinone)	46.8/5.61	cyt, MLS	cyt
FTH_1763	NADH dehydrogenase (ubiquinone)	47.59/7.06	cyt, MLS	cyt
FTH_1824	Conserved hypothetical protein	20.32/6.64	CM	TMH
FTH_1830	Cell division protein FtsZ	39.8/4.76	cyt, MLS	cyt
FTH_1831	Cell division protein FtsA	44.80/5.20	cyt	cyt
FTH_1875	N-(5'-Phosphoribosyl)anthranilate isomerase	51.32/8.86	cyt	cyt

^a The accession number in the genome sequence of *F. tularensis* subsp. *holarctica* OSU18. ^b Theoretical molecular weight/theoretical pI. ^c Prediction of the protein localization using the PSORTb program, cyt—cytoplasmic, CM cytoplasmic membrane, OM—outer membrane, PP—periplasm, EC—extracellular space, ?—unknown localization, MLS—multiple localization sites. ^d Prediction of lipoproteins (SPII cleavage site II) and SPI (cleavage site I) using the LipoP algorithm, TMH—transmembrane domain.

Table 3 List of *F. tularensis* proteins isolated on magnetic hydrazide-PHEMA microspheres

Gene locus ^a	Protein name	(<i>M_w</i> ^b /kDa)/pI	PSORTb ^c	LipoP ^d
FTH_0039	Putative uncharacterized protein	15.30/5.16	?	SPII
FTH_0038	Probable multidrug efflux pump	37.24/9.17	CM	TMH
FTH_0066	30S ribosomal protein S20	10.15/10.96	cyt	cyt
FTH_0091	Putative uncharacterized protein	13.77/5.03	cyt	cyt
FTH_0105	Intracellular growth locus C protein	22.38/5.65	?	cyt
FTH_0108	Conserved protein PdpC	156.00/9.17	cyt	cyt
FTH_0124	FeoB family ferrous iron (Fe ²⁺) uptake protein	81.46/8.55	CM	cyt
FTH_0159	Universal stress protein	30.22/6.10	cyt	cyt
FTH_0172	Inner membrane protein oxaA	61.95/8.74	CM	SPI
FTH_0187	Cytochrome o ubiquinol oxidase subunit I	76.20/8.58	CM	TMH
FTH_0219	Ribosomal protein S2	26.42/8.94	cyt	cyt
FTH_0227	Ribosomal protein S12	13.81/11.58	cyt	cyt
FTH_0228	Ribosomal protein S7	17.82/10.82	cyt	cyt
FTH_0230	Ribosomal protein S10	11.90/9.79	cyt	cyt
FTH_0231	Ribosomal protein L3	22.31/9.65	cyt	cyt
FTH_0232	50S ribosomal protein L4	22.55/10.29	cyt	cyt
FTH_0233	Ribosomal protein L23	11.14/9.82	cyt	cyt
FTH_0234	Ribosomal protein L2	30.40/11.54	cyt	cyt
FTH_0236	Ribosomal protein L22	12.20/10.80	cyt	cyt
FTH_0237	Ribosomal protein S3	24.88/10.10	cyt	cyt
FTH_0238	Ribosomal protein L16	15.71/11.43	cyt	cyt
FTH_0239	Ribosomal protein L29	7.80/10.34	cyt	cyt
FTH_0241	Ribosomal protein L14	13.23/10.51	cyt	cyt
FTH_0242	50S ribosomal protein L24	11.48/9.73	cyt	cyt
FTH_0243	Ribosomal protein L5	20.00/9.75	cyt	cyt
FTH_0244	30S ribosomal protein S14	11.72/10.36	cyt	cyt
FTH_0245	Ribosomal protein S8	14.41/9.38	cyt	cyt
FTH_0247	Ribosomal protein L18	13.04/9.97	cyt	cyt
FTH_0248	Ribosomal protein S5	17.56/9.99	cyt	cyt
FTH_0249	50S ribosomal protein L30	6.87/10.09	cyt	cyt
FTH_0250	Ribosomal protein L15	15.10/10.24	cyt	cyt
FTH_0251	Preprotein translocase subunit secY	48.46/10.01	CM	TMH
FTH_0253	Ribosomal protein S13	13.38/11.54	cyt	cyt
FTH_0254	Ribosomal protein S11	13.76/10.85	cyt	cyt
FTH_0255	Ribosomal protein S4	23.26/9.85	cyt	cyt
FTH_0257	Ribosomal protein L17	16.78/10.72	cyt	cyt
FTH_0280	Chaperone DnaJ	33.67/8.93	cyt	cyt
FTH_0295	Acetyl-CoA carboxylase α subunit	35.44/8.07	cyt	cyt
FTH_0310	Pyruvate dehydrogenase (acetyl-transferring)	100.27/5.65	cyt	cyt
FTH_0311	Dihydrolypoyllysine-residue acetyltransferase	56.79/4.79	CM	cyt
FTH_0312	Dihydrolypoyl dehydrogenase	50.53/5.87	cyt	cyt
FTH_0403	Putative uncharacterized protein	32.50/8.46	cyt	cyt
FTH_0463	Soluble lytic murein transglycosylase	76.93/9.09	PP	SPI
FTH_0515	Cell division topological specificity factor protein MinE	10.18/8.03	cyt	cyt
FTH_0516	Septum site-determining protein MinD	30.8/6.85	cyt, MLS	cyt
FTH_0519	Ribosomal protein L28	8.94/11.06	cyt	cyt
FTH_0540	(3 <i>R</i>)-Hydroxymyristoyl-[acyl-carrier-protein] dehydratase	18.15/6.83	cyt	cyt
FTH_0570	Putative uncharacterized protein	19.79/9.74	?	SPI
FTH_0585	Acyl-CoA dehydrogenase	84.19/8.45	?	cyt
FTH_0586	Long-chain-fatty-acid-CoA ligase	63.07/7.57	CM	cyt
FTH_0589	Putative uncharacterized protein	33.26/9.53	CM	TMH
FTH_0592	dTDP-glucose 4,6-dehydratase	65.71/8.17	CM	TMH
FTH_0593	Galactosyltransferase	23.78/10.21	CM	SPI
FTH_0594	UDP-glucose 4-epimerase	29.96/9.46	?	SPI
FTH_0595	Galacturonosyltransferase	41.60/9.39	cyt	cyt
FTH_0604	Glycosyltransferase	32.98/6.51	cyt	cyt
FTH_0620	Probable bacterioferritin	18.46/5.83	cyt	cyt
FTH_0646	Conserved hypothetical protein	14.93/8.92	?	SPII
FTH_0689	Probable multidrug resistance efflux pump	37.83/9.36	?	cyt
FTH_0719	Ribonuclease E	95.95/7.12	cyt	cyt
FTH_0792	Type IV pilus assembly protein	23.00/9.58	?	SPI
FTH_0799	Bifunctional 1-pyrroline-5-carboxylate dehydrogenase/proline dehydrogenase	150.39/7.97	cyt	cyt
FTH_0800	APC family amino acid-polyamine-organocation transporter	55.00/9.27	CM	TMH
FTH_0837	RND superfamily resistance-nodulation-cell division antiporter	69.67/9.52	CM	TMH/SPI
FTH_0881	Conserved hypothetical protein	54.87/8.16	CM	TMH
FTH_0886	Probable membrane protease subunit HflK	40.42/8.97	cyt	cyt
FTH_0887	Membrane protease subunit HflC	34.59/9.97	?	cyt
FTH_0905	Ferritin protein	19.07/4.75	cyt	cyt
FTH_0928	50S ribosomal protein L25	10.90/9.26	cyt	cyt
FTH_1002	Ribosomal protein L9	16.45/5.55	cyt	cyt
FTH_1026	Ribosomal protein S21	7.84/11.4	cyt	cyt

Table 3 (Contd.)

Gene locus ^a	Protein name	(M _w ^b /kDa)/pI	PSORTb ^c	LipoP ^d
FTH_1071	Probable thioredoxin family protein	39.55/4.67	?	SPII
FTH_1107	Inositol-phosphate phosphatase	28.87/8.87	cyt	cyt
FTH_1112	3-Oxoacyl-[acyl-carrier-protein] synthase	44.02/5.72	cyt	cyt
FTH_1117	Fatty acid/phospholipid synthesis protein PlsX	37.84/9.62	cyt	cyt
FTH_1118	50S ribosomal protein L32	6.88/10.2	cyt	cyt
FTH_1162	Ribosomal protein S9	10.09/10.35	cyt	cyt
FTH_1163	Ribosomal protein L13	15.94/10.03	cyt	cyt
FTH_1167	Chaperone protein dnaK (HSP70)	69.18/4.62	cyt	cyt
FTH_1179	Cardiolipin synthase	54.86/8.37	CM	cyt
FTH_1278	Putative uncharacterized protein	38.80/8.35	?	SPI
FTH_1281	Acetyl-CoA carboxylase β subunit	33.65/8.96	cyt	cyt
FTH_1354	ATP-dependent RNA helicase	64.01/8.91	cyt	cyt
FTH_1366	Ribosomal protein L20	13.34/10.81	cyt	cyt
FTH_1377	Putative uncharacterized protein	44.26/9.77	?	TMH
FTH_1379	Probable capsule biosynthesis protein	44.90/7.41	CM	TMH
FTH_1424	M41 family endopeptidase FtsH/HflB	70.74/5.42	CM	TMH
FTH_1443	Bifunctional FAD/FMN dehydrogenase/Fe-S oxidoreductase	114.57/8.64	cyt	cyt
FTH_1462	MFS family major facilitator transporter, glycerol-3-phosphate uniporter	48.02/8.51	CM	cyt
FTH_1487	Polyribonucleotide nucleotidyltransferase	75.53/5.68	cyt	cyt
FTH_1488	Ribosomal protein S15	10.36/10.13	cyt	cyt
FTH_1489	Peptidoglycan glycosyltransferase	62.73/8.77	CM	TMH
FTH_1492	Probable acyl-coenzyme A synthetase	78.58/9.04	CM	cyt
FTH_1503	Conserved hypothetical protein	69.85/10.14	CM	TMH
FTH_1536	Conserved hypothetical protein	25.35/6.96	?	cyt
FTH_1558	Glycosyltransferase, group 2 family protein	36.08/8.69	CM	cyt
FTH_1599	Conserved hypothetical protein	49.32/4.33	?, MLS	SPI
FTH_1612	RND family efflux transporter, MFP subunit	50.09/10.12	CM	cyt
FTH_1613	RND superfamily resistance-nodulation-cell division:proton (H ⁺) antiporter	112.50/5.40	CM	TMH
FTH_1617	Conserved hypothetical protein	38.46/9.50	CM	SPI
FTH_1651	Chaperone GroEL	57.40/4.72	cyt	cyt
FTH_1662	Putative uncharacterized protein	23.83/9.93	?	cyt
FTH_1674	Ribosomal protein L19	13.31/10.53	cyt	cyt
FTH_1686	50S ribosomal protein L10	18.73/9.54	cyt	cyt
FTH_1687	Ribosomal protein L1	24.63/9.50	cyt	cyt
FTH_1688	50S ribosomal protein L11	15.27/9.49	cyt	cyt
FTH_1691	Elongation factor Tu	43.39/4.87	cyt	cyt
FTH_1696	Glycerol-3-phosphate dehydrogenase	57.78/8.36	?, MLS	cyt
FTH_1708	Aconitate hydratase	102.70/5.30	cyt	cyt
FTH_1719	Dihydrolipoyllysine-residue succinyltransferase	52.75/4.89	cyt	cyt
FTH_1720	Oxoglutarate dehydrogenase (succinyl-transferring)	105.67/6.01	cyt	cyt
FTH_1721	Succinate dehydrogenase	26.57/8.18	CM	cyt
FTH_1722	Succinate dehydrogenase	65.86/6.14	CM	cyt
FTH_1731	ATP synthase epsilon chain (ATP synthase F1 sector epsilon subunit)	15.74/6.75	?	cyt
FTH_1732	H(+)-transporting two-sector ATPase	49.87/4.89	cyt	cyt
FTH_1733	H(+)-transport 2-sector ATPase	33.19/8.87	cyt, MLS	cyt
FTH_1734	H(+)-transporting two-sector ATPase	55.54/4.68	cyt	cyt
FTH_1735	H(+)-transporting two-sector ATPase	19.20/6.11	cyt	cyt
FTH_1760	NADH dehydrogenase (ubiquinone)	87.36/5.09	cyt, MLS	cyt
FTH_1761	NADH dehydrogenase (ubiquinone)	46.28/5.55	cyt, MLS	cyt
FTH_1762	NADH dehydrogenase (ubiquinone)	18.17/5.00	cyt	cyt
FTH_1763	NADH dehydrogenase (ubiquinone)	47.59/7.06	cyt, MLS	cyt
FTH_1764	NADH-quinone oxidoreductase subunit C	25.20/6.95	cyt, MLS	cyt
FTH_1794	Protein-L-isoaspartate (D-aspartate) O-methyltransferase	23.20/5.25	cyt	cyt
FTH_1830	Cell division protein FtsZ	39.75/4.49	cyt, MLS	cyt
FTH_1837	Conserved hypothetical protein	21.08/7.07	CM	cyt
FTH_1855	ABC superfamily ATP binding cassette transporter, binding protein	33.82/5.74	?	SPI
FTH_1874	Heat shock protein	16.74/5.58	cyt	cyt

^a The accession number in the genome sequence of *F. tularensis* subsp. *holarctica* OSU18. ^b Theoretical molecular weight/theoretical pI. ^c Prediction of the protein localization using the PSORTb program, cyt—cytoplasmic, CM cytoplasmic membrane, OM—outer membrane, PP—periplasm, EC—extracellular space, ?—unknown localization, MLS—multiple localization sites. ^d Prediction of lipoproteins (SPII cleavage site II) and SPI (cleavage site I) using the LipoP algorithm, TMH—transmembrane domain.

Furthermore, 91 proteins, both previously and not previously identified, were common to both tested hydrazide-modified microspheres (Fig. 3).

It should be noted that the glycosylation status of all identified, hydrazide-captured proteins was not confirmed yet.

Nevertheless, among the proteins isolated from all three biological replicates using SiMAG-Hydrazide and from two replicates using magnetic hydrazide-PHEMA microspheres was protein PilA. PilA, the component of type IV pili, was the first *F. tularensis* protein being discovered as glycosylated.^{31,32}

Table 4 List of hydrazide-isolated *F. tularensis* proteins that were previously identified¹⁰

Gene locus ^a	ProQ Emerald ^b	DIG Glycan ^c	Lectin affinity chromatography
FTH_0039	No	No	ConA, DSA, PNA, SBA, SNA
FTH_0117	No	No	SBA
FTH_0139	No	No	DSA, SBA
FTH_0159	No	SNA	ConA, DSA, PNA, SBA, SNA
FTH_0172	No	No	ConA, DSA, SBA
FTH_0184	No	No	DSA, SBA
FTH_0186	No	No	DSA, SBA
FTH_0187	No	No	PNA, SBA, SNA
FTH_0219	No	No	ConA, DSA, PNA, SBA
FTH_0228	No	No	ConA, DSA, PNA, SNA
FTH_0232	No	No	DSA, PNA, SBA
FTH_0234	No	No	DSA, PNA
FTH_0236	No	No	DSA, PNA, SBA, SNA
FTH_0251	No	No	SBA
FTH_0253	No	No	ConA, DSA, SNA
FTH_0257	No	No	ConA, DSA, SBA
FTH_0295	No	No	ConA, DSA, PNA, SBA, SNA
FTH_0310	No	No	ConA, DSA, PNA, SBA, SNA
FTH_0311	No	SNA	DSA, PNA, SBA, SNA
FTH_0312	No	No	ConA, DSA, PNA, SNA
FTH_0334	No	No	ConA, DSA, PNA, SNA
FTH_0357	Yes	No	ConA, DSA, PNA, SBA, SNA
FTH_0384	Yes	No	ConA, DSA, PNA, SBA, SNA
FTH_0447	No	No	DSA, SBA
FTH_0516	No	MAA	No
FTH_0539	No	SNA	SBA
FTH_0541	No	No	SBA
FTH_0570	No	No	ConA, DSA, PNA, SBA, SNA
FTH_0585	No	No	DSA, SBA
FTH_0589	No	No	DSA, SBA
FTH_0592	No	No	ConA, DSA, SBA, SNA
FTH_0593	No	No	ConA, DSA, SBA
FTH_0604	No	No	ConA, DSA, SBA
FTH_0620	No	No	ConA, DSA, PNA, SNA
FTH_0628	No	No	DSA, SBA
FTH_0646	Yes	No	No
FTH_0719	No	No	DSA, PNA, SBA
FTH_0799	No	No	SBA
FTH_0836	No	No	ConA, DSA, SBA, SNA
FTH_0837	No	No	DSA, SBA
FTH_0886	No	No	DSA, SBA
FTH_0887	No	No	DSA, SBA
FTH_1071	Yes	No	ConA, DSA, SBA, SNA
FTH_1078	No	No	SBA
FTH_1112	No	SNA	DSA
FTH_1117	No	No	DSA, SBA
FTH_1167	No	SNA	ConA, DSA, SNA
FTH_1216	No	No	DSA, SBA, SNA
FTH_1377	No	No	ConA, DSA, SBA, SNA
FTH_1379	No	No	DSA, SBA
FTH_1424	No	No	DSA, SBA
FTH_1462	No	No	SBA
FTH_1503	No	No	ConA, DSA, PNA, SBA, SNA
FTH_1536	No	No	DSA, SBA
FTH_1558	No	No	DSA, SBA
FTH_1599	No	No	DSA, SBA
FTH_1609	No	No	DSA, SBA
FTH_1612	No	No	ConA, DSA, SBA
FTH_1617	No	No	ConA, DSA, PNA, SNA
FTH_1651	No	No	ConA, DSA, PNA, SBA, SNA
FTH_1662	No	No	DSA, SBA
FTH_1686	No	No	DSA, PNA, SBA, SNA
FTH_1691	No	No	DSA, SBA, SNA
FTH_1696	No	No	DSA, SBA
FTH_1708	No	No	ConA, DSA, PNA, SBA, SNA
FTH_1719	No	No	ConA, DSA, PNA, SBA, SNA
FTH_1721	No	MAA, PNA	ConA, DSA, PNA, SBA, SNA
FTH_1722	No	No	ConA, DSA, PNA, SBA, SNA
FTH_1726	No	No	SBA
FTH_1732	No	No	ConA, DSA, PNA, SBA, SNA
FTH_1733	No	No	DSA, SBA

Table 4 (Contd.)

Gene locus ^a	ProQ Emerald ^b	DIG Glycan ^c	Lectin affinity chromatography
FTH_1734	No	No	DSA, PNA, SBA, SNA
FTH_1735	No	No	DSA, SBA
FTH_1736	No	No	DSA, SBA
FTH_1760	No	No	ConA, DSA, SBA, SNA
FTH_1761	No	SNA	ConA, DSA, SBA
FTH_1763	No	No	DSA, SBA, SNA
FTH_1764	No	No	ConA, DSA, SNA
FTH_1830	No	SNA	ConA, DSA, PNA, SBA, SNA
FTH_1837	No	No	DSA, PNA, SBA, SNA
FTH_1855	No	SNA	ConA, DSA, SBA

^a The accession number in the genome sequence of *F. tularensis* subsp. *holarctica* OSU18. ^b ProQ Emerald carbohydrate-specific staining. ^c DIG Glycan Differentiation kit.

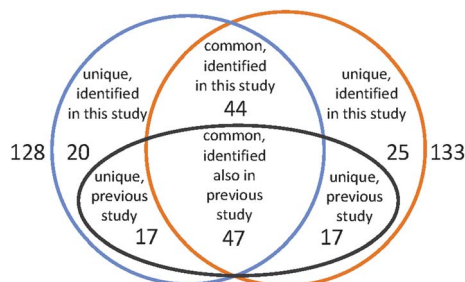


Fig. 3 Comparison of magnetic hydrazide-PHEMA microspheres (blue circle) and SiMAG-Hydrazide (orange circle) for glycoprotein isolations from *F. tularensis* with previously identified *F. tularensis* putative glycoproteins (black circle).

Additionally, an immunoreactive protein FTH_1071, for which the glycosylation status was recently verified (Balonova *et al.*, manuscript in preparation), was isolated on both types of magnetic microspheres.

Conclusions

The commercial SiMAG-Hydrazide microparticles with a silica shell chemically modified with hydrazide groups have recently been proven successful for capturing human platelet glycoproteins¹⁸ and for oriented immobilization of glycosylated anti-ovalbumin IgG antibodies.³³ The primary advantage of magnetic microspheres is their ability to be quickly and easily separated from complex mixtures using a magnet. In the present study, new magnetic hydrazide-PHEMA microspheres were synthesized by reaction of hydrazine with macroporous P(HEMA-Ac-co-MCMEMA-co-EDMA) microspheres prepared by the multi-step swelling and polymerization method. The method has an advantage that it provides monodisperse microspheres. Magnetic hydrazide-PHEMA microspheres were then compared with commercial SiMAG-Hydrazide particles for capturing glycoproteins of bacterial origin, such as *F. tularensis* glycoproteins. Almost identical binding capacity of both magnetic microspheres was observed with respect to the number of identified putative glycoproteins. In total, 50% of all identified *F. tularensis* putative glycoproteins have already been identified in a previous study.¹⁰

Suitability of hydrazide groups bound to magnetic microspheres as a carrier is obvious from the molecular weight

distributions of identified proteins. It is apparent from Fig. 4 that magnetic microspheres, in general, allow isolation of proteins regardless of their molecular weight, and the molecular sieving effect frequently observed with non-magnetic carriers based on agarose is absent. Furthermore, hydrazide groups attached to the microspheres offer two advantages for glycoprotein isolation compared with the conventional lectin affinity chromatography. First, detergents solubilizing strongly hydrophobic membrane proteins present in the cell lysates can be securely used and, second, harsher washing solvents, such as organic and chaotropic agents, at high concentrations can be applied prior to proteolytic digestion of trapped glycoproteins in order to minimize co-elution of nonspecifically adsorbed non-glycoproteins.

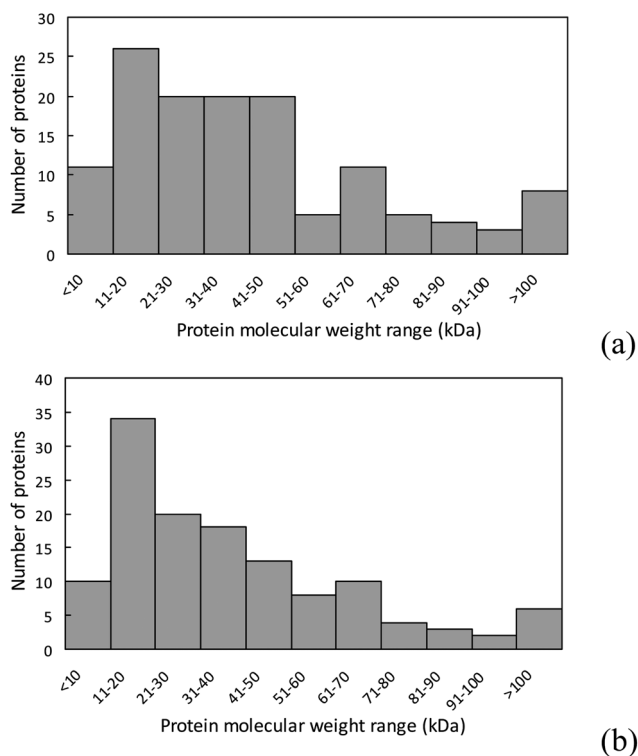


Fig. 4 Molecular weight distribution of identified proteins isolated on (a) magnetic SiMAG-Hydrazide microspheres and (b) magnetic hydrazide-PHEMA microspheres.

On the other hand, the information about glycosylation of the isolated glycoproteins is lost during hydrazide-based capturing as the reaction between the glycan and hydrazide group is irreversible and enzymatic release of peptides from *F. tularensis* glycoproteins through PNGase F is not possible. Therefore, the present technique is convenient exclusively for identification of glycosylated proteins enabling an isolation of the entire glycoprotein pool in a single analysis. Additionally, the hydrazide-based approach for enrichment of glycoproteins can be utilized as another fractionation technique to simplify complexity of the protein mixture for subsequent mass spectrometric analysis.

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