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**PAPER**

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

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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.

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Nonglycosylated proteins are removed by washing, while captured glycoproteins are proteolytically digested. Non-glycosylated 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 microspheres[18] 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. $FeCl_2 \cdot 4H_2O$, 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 $CO_2$ 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 $CO_2$ 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 $FeCl_2$ 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 $NH_4OH$ 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, Diskinson, 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 ($OD_{600nm} = 0.1$) and grown until the late logarithmic growth phase of bacteria ($OD_{600nm} = 0.8$). Bacterial cells were collected by centrifugation at 9000*g* 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 12 600*g* for 30 min at 4 °C. Benzonase nuclease (361 U $\mu l^{-1}$) 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 115 000*g* 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 115 000*g* 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 10 000*g* 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 dithiotreitol 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 μg 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 preconcentrated on a micro-precolumn C18 cartridge (300 μm i.d. × 5 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 μm i.d. × 150 mm) packed in-house with Magic C18AQ particles (200 Å, 3 μm) 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 software[22] 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 localization[23] 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 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 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.

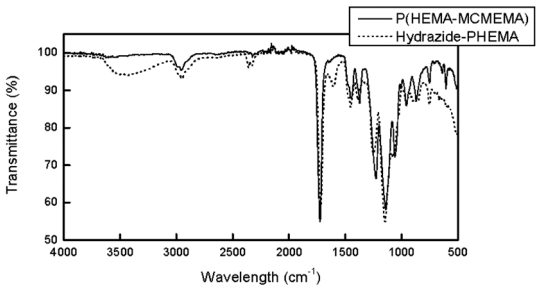


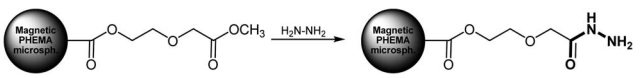

**Fig. 1** ATR-FTIR spectra of P(HEMA-Ac-*co*-MCMEMA-*co*-EDMA) microspheres (—) before and (···) after hydrolysis with 0.5 N NaOH.

### 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 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, $Fe(OH)_2$ is formed and oxidized by atmospheric oxygen to $Fe(OH)_3$, which, in combination with $Fe(OH)_2$, 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.

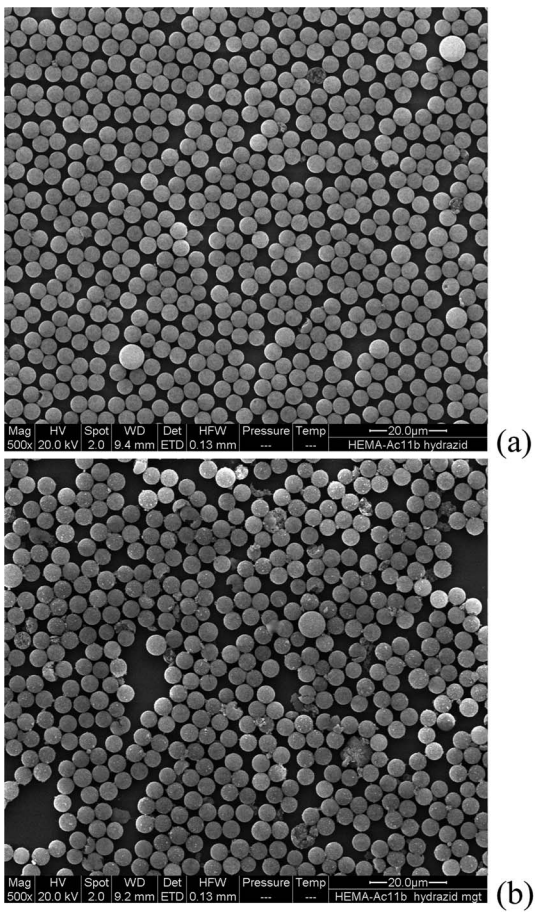



**Fig. 2** SEM of (a) hydrazide-PHEMA microspheres and (b) magnetic hydrazide-PHEMA microspheres (13.1 wt% Fe).

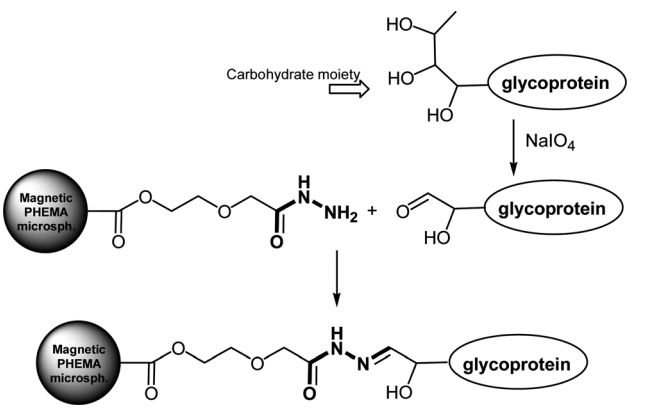
2-acetoxyethyl units in the P(HEMA-Ac-*co*-MCMEMA-*co*-EDMA) microspheres to free 2-hydroxyethyl groups of PHEMA. The hydrazide-PHEMA particle size and size distribution were not changed during the formation of iron oxides as documented by the SEM micrograph (Fig. 2b).

### 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 *N*-acyltransferase | 35.8/5.80 | cyt | cyt |
| FTH_0540 | (3*R*)-Hydroxymyristoyl-[acyl-carrier-protein] dehydratase | 18.15/6.83 | cyt | cyt |
| FTH_0541 | Acyl-[acyl-carrier-protein]-UDP-*N*-acetylglucosamine *O*-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 | ($M_w$[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 | Dihydrolipoyllysine-residue acetyltransferase | 56.79/4.79 | CM | cyt |
| FTH_0312 | Dihydrolipoyl 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*R*)-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 identified[10]

| 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.

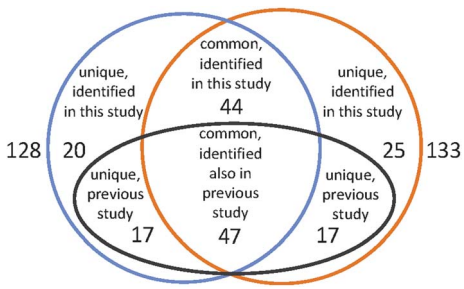



**Fig. 3** Comparison of magnetic hydrazide-PHEMA microspheres (blue circle) and SiMAG-Hydrazide (orange circle) for glycoprotein isolations from *F. tularensis* with previously identified *F. tularensis* putative glycoproteins (black circle).

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 glycoproteins[18] 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.

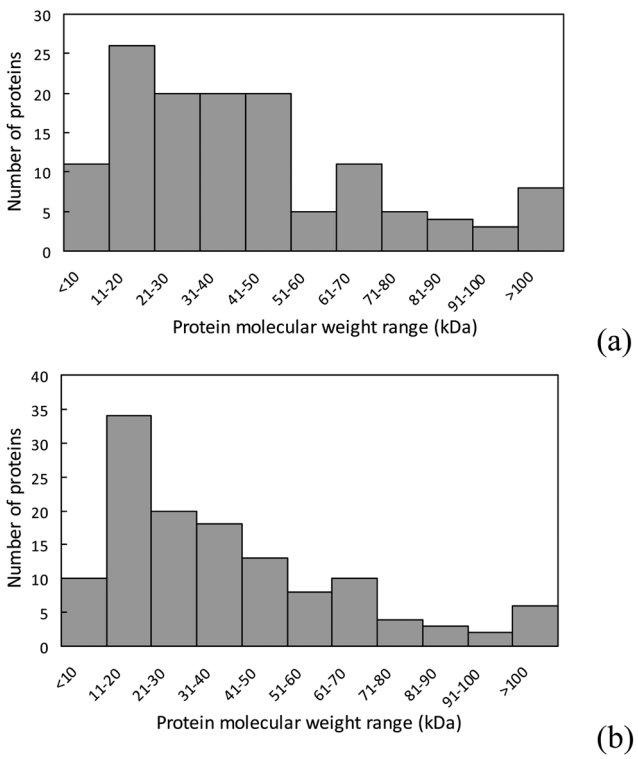



**Fig. 4** Molecular weight distribution of identified proteins isolated on (a) magnetic SiMAG-Hydrazide microspheres and (b) magnetic hydrazide-PHEMA microspheres.

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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