Despite numerous studies, isolating pure preparations of extracellular vesicles (EVs) has proven challenging. Here, we compared ion-exchange chromatography (IEC) to the widely used sucrose density gradient (SDG) centrifugation method for the purification of EVs. EVs in bulk were isolated from pooled normal human amniotic fluid (AF) by differential centrifugation followed by IEC or sucrose density gradient separation. The purity of the isolated EVs was evaluated by electrophoresis and lectin blotting/immuno blotting to monitor the distribution of total proteins, different EVs markers, and selected N-glycans. Our data showed efficient separation of negatively charged EVs from other differently charged molecules, while comparative profiling of EVs using SDG centrifugation confirmed anion-exchange chromatography is advantageous for EV purification. Finally, although this IEC-based method was validated using AF, the approach should be readily applicable to isolation of EVs from other sources as well.
Materials and methods

Amniotic fluid samples
AF samples were obtained from pregnant women who had undergone routine amniocentesis at 16–18 weeks gestation. Amniocenteses were performed due to advanced maternal age or at maternal request. After removal of cells needed for routine karyotype analysis, leftover AF, which is usually discarded, was de-identified and used for this research. Our investigation involved the use of existing human specimens and therefore is not considered research on human subjects for which informed consent is required according to guidelines of “The rules of procedures of the Ethics Committee of INEP” (No. 02–832/1). The study was approved by the local Ethics Committee of INEP, No. 03–359/1, on May 13th, 2015. Only samples with a normal fetal karyotype and no pathologies were used. Specimens were obtained from women with an average age of 36.5 years. The AF samples (16 mL each) were pooled to average out the heterogeneity of individual samples. Three pools, each consisting of six different samples, were examined.

Isolation of extracellular vesicles (EVs)
AF pools were differentially centrifugated at 300 × g (10 min), 3000 × g (20 min), 17,000 × g (25 min), and 100,000 × g (2 h), (Ti 50.2 rotor, k-factor = 157.7) (Optima L-90K ultracentrifuge; Beckman Coulter, Indianapolis, IN). The final pellet, enriched in EVs (P100), was resuspended in 1 mL 0.05M Tris–HCl buffer, pH 7.6, by pipetting up and down 15 times and vortexing for 1 min. Protein concentration was determined using the BCA Protein Quantification Kit (ab102536; Abcam, Cambridge, UK).

Separation of amniotic fluid EVs using a sucrose density gradient
P100 preparations were separated by SDG (0.5 M, 0.7 M, 0.9 M, 1.1 M, and 1.3 M sucrose) centrifugation at 100,000 × g for 2 h (SW41Ti rotor; k factor = 256.6), as described earlier (17,18). Twelve fractions (1 mL) were collected from the top of the tubes and subjected to acetone precipitation (18). The collected proteins were resuspended in an equal volume of 0.05 M PBS and used for further analysis.

Transmission electron microscopy
Transmission electron microscopy (TEM) of EVs was performed as described previously (19). Images were collected using a Philips CM12 electron microscope (Philips, Eindhoven, The Netherlands).

Concanavalin A binding assay
Charge-resolved IEC fractions were immobilized on microwell plates (Thermo Scientific Nunc, Roskilde, Denmark) in 0.05 M carbonate buffer pH 9.5 (50 µl well) and incubated for 18 h at 4°C. The Con A binding assay was then performed as described earlier (19).

Dot blot of EV markers
A nitrocellulose membrane (Thermo Scientific, Rockford, IL) was dotted with IEC fractions (2 µl) and incubated with the following: (i) blocking solution containing 3% BSA for 2 h at RT; (ii) mouse monoclonal antibody to a corresponding EV marker: anti-CD63 antibody, clone TS63 (ab59479; Abcam; RRID:AB_940915) (5 µg/mL) for 18 h at 4°C; anti-CD9 antibody, clone CGS12A (EX201-100; Cell Guidance Systems, Cambridge, UK) (1 µg/mL) for 18 h at 4°C; anti-CD81 Ab, clone CGS36K (EX203-100; Cell Guidance Systems) (1 µg/mL) for 18 h at 4°C; (iii) biotinylated goat anti-mouse IgG (BA-9200; Vector Laboratories; RRID:AB_2336171) (0.75 µg/mL) for 30 min at RT; and (iv) avidin/biotinylated-horseradish peroxidase (HRP) (#PK-6100, Vector Laboratories) for 30 min at RT. Between each step, the membrane was rinsed 3x with 0.05 M PBS, pH 7.2 for 10 min. Blots were visualized using Pierce ECL Western Blotting Substrate (#32106, Thermo Scientific). TotalLab Software, version 2.00 (Amersham Biosciences, Buckinghamshire, UK) was employed for densitometry analysis.

SDS-PAGE
Samples (as isolated) were prepared in reducing Laemmli buffer and separated on a 10% gel (20). Gels were calibrated using broad-range SDS-PAGE standards, and proteins were stained using a silver stain kit (#161–0443, Bio-Rad, Hercules, CA).

Lectin blotting and immunoblotting
Charge-resolved fractions of EVs, pooled and concentrated by ultrafiltration, or acetone-precipitated sucrose gradient fractions, were resolved on 10% gels and transferred to a nitrocellulose membrane. Membranes were blocked with 3% BSA and subjected to lectin blotting or immunoblotting. For lectin blots, membranes were incubated with the biotinylated lectins Con A and SNA (Sambucus nigra agglutinin; Vector Laboratories) at a concentration of 1 µg/mL for 1 h at RT. Bound lectins were detected by incubation with avidin/biotinylated-HRP for 30 min at RT. Immunoblots were performed as described elsewhere (19). Human
IgG was detected using biotinylated goat anti-human IgG heavy chain (H) antibodies (BA-3080; Vector Laboratories; RRID:AB_2336154) (2 µg/mL) and avidin/biotinylated-HRP. Albumin was detected using rabbit anti-human albumin (Calbiochem, San Diego, CA) (1:5000) and HRP-labeled sheep anti-rabbit antibodies (AbD Serotec, Kidlington, UK) (0.1 µg/mL). Pierce ECL Western blotting substrate was used for visualization.

Results and discussion

As an initial step in protocols for isolation of EVs, differential centrifugation is known to yield concentrated EVs in the 100,000 × g pellet, together with co-isolated contaminants (21). When applied to AF (Figure 1), soluble proteins (S100; 100,000 × g supernatant) and those from the matching pellet (P100; 100,000 × g pellet) exhibited partially overlapping patterns in the lower molecular mass range (<80 kDa), where albumin (66 kDa) and IgG (seen as 55 kDa heavy chain and 25 kDa light chain) are expected to migrate as known contaminants/associated proteins (http://microvesicles.org/gene_summary?gene_id = 213) (21–23). In contrast, high molecular mass components, which could originate from different types of protein complexes or aggregates, or from aEVs themselves, were enriched in the P100 pellet.

Bulk aEVs (P100) separated by centrifugation in an SDG, which is commonly used for the removal of unwanted co-isolated proteins, and visualized in an SDS-PAGE gel are shown in Figure 2. The electrophoretic patterns of proteins in the lower sucrose density fractions (Fractions 2–5) were similar to that of the top of the gradient (Fraction 1), where soluble proteins are supposed to remain (Figure 2A). In the higher sucrose density fractions, slight differences in the removal of particular proteins of lower molecular mass (<40 kDa) were noticeable. Specifically, albumin was found to decrease across those fractions, as shown by the albumin-immunoreactive band intensity (Figure 2B), and the same was true for the IgG-immunoreactive bands (Figure 2C). In addition, the distribution of CD63 (Figure 2D), a common marker for EVs, indicated that aEVs were concentrated mainly in the low density sucrose gradient fractions (i.e. Fractions 2–4), but were also present in a mid-density fraction (Fraction 6).

Separation of aEVs using density gradient centrifugation has been reported (17,24,25), but no data were given on the yield or the protein composition of the isolated populations, including the presence of known contaminants.

Our results using density gradient centrifugation showed considerable overlap of the major aEV-containing fractions with the top gradient fraction with respect to the total protein/albumin/IgG distribution. This was in agreement with previously reported data (26), but it has not yet been resolved whether this is a general phenomenon due to the nature of EVs themselves or if it is related to the limitations of current purification methods.

Pure preparations of isolated EVs are necessary to determine whether an observed property/biological activity is intrinsic to the EVs. This is of particular importance for proper clinical and therapeutic applications in terms of exploring their multifunctional potential or diminishing the side effects of co-purified components. Given the importance of purity for downstream analysis, we examined IEC as an alternative purification step to sucrose gradient centrifugation. As negatively charged entities in a mixture of differently charged molecules, aEVs were resolved on an anion-exchange column into five fractions (Figure 3). Con A lectin, previously shown to interact strongly with EVs (19,27), was used to monitor elution (Figure 3A). Fractions eluted with 0.2 M NaCl and 1 M NaCl were strongly Con A-reactive, while weaker reactivity was observed for the non-bound fraction and the fractions eluted with 0.05 M NaCl and 0.1 M NaCl.

The EV marker proteins CD63 and CD81 were found in the fractions eluted with 0.2 M NaCl, and they overlapped also in the leading fractions eluted with 1 M NaCl (Figure 3B). In addition, low but detectable immunoreactivity to CD9 was observed in both of these charge-resolved populations. The presence of EVs was additionally confirmed by TEM (Figure 3C). Thus, fractions eluted with 0.2 M NaCl contained smaller EVs, ranging 60–100 nm (Figure 3C, Panel 1), whereas in the fraction eluted by 1 M NaCl, they were larger, ranging 90–220 nm (Figure 3C, Panel 2e). This indicated that in spite of the use of high salt concentrations, the separated aEVs maintained their biochemical and physical characteristics, as previously observed with human urinary EVs and mouse fibroblast L cell EVs under comparable conditions (19,28).

IEC separates and concentrates different components of heterogeneous samples into distinct fractions. In contrast, some currently used methods lead to sample dilution, requiring consecutive concentration steps or removal of separation media such as sucrose, all of which causes loss of EVs. Generally, it has been reported that the yield of EVs can vary considerably, from 20%–60% for size
Figure 3. Separation of amniotic fluid extracellular vesicles (afEVs) using ion-exchange chromatography (IEC). afEVs in bulk (100,000 × g pellet) were subjected to IEC on a Sephadex DEAE A-50 column eluted with 50 mM Tris–HCl buffer, pH 7.6 (a) containing increasing salt concentrations: 50 mM NaCl (b), 100 mM NaCl (c), 200 mM NaCl (d), and 1 M NaCl (e). Fractions of 2 mL were collected. (A) Elution of charge-resolved afEV fractions was monitored by measuring binding of solid phase-immobilized fractions to Con A (concanavalin A) lectin. OD: optical density. (B) Elution of afEVs was monitored by measuring the reactivity of dot blot-immobilized fractions with anti-CD63, anti-CD9, and anti-CD81 antibodies. A representative profile obtained by densitometry of dot signal intensities is shown. ADU: arbitrary densitometry units. (C). Transmission electron micrographs of fractions eluted with 200 mM NaCl (1) and 1 M NaCl (2). (D) Selected samples, as indicated by points in (B), from each charge-resolved afEV fraction were subjected to 10% SDS-PAGE and silver stained. Arrows and numbers indicate the position of molecular mass standards in kDa. The arrowhead indicates the position of albumin.

Figure 4. Glycan patterns of charge-resolved amniotic fluid extracellular vesicles (afEVs). Charge-resolved fractions of afEVs were pooled, concentrated, and subjected to 10% SDS-PAGE following lectin blotting or immunoblotting. (A) Representative patterns of bands reactive with concanavalin A (Con A) lectin. (B) Representative patterns of bands reactive with SNA (Sambucus nigra agglutinin). (C) Representative pattern of bands reactive with anti-human immunoglobulin G (IgG) heavy chain (H). Numbers indicate the position of molecular mass standards in kDa. Pooled and concentrated fractions eluted with 50 mM Tris–HCl buffer, pH 7.6 (a), containing increasing salt concentrations: 50 mM NaCl (b), 100 mM NaCl (c), 200 mM NaCl (d), and 1 M NaCl (e).
exclusion chromatography to 2%–80% for ultracentrifugation, and this also depends on the quality of the source material (13).

For IEC, the recovery of total protein (input) was 80%. The distribution of recovered proteins was 0.6% in the non-bound fraction, 3.2% in the fraction eluted with 0.05 M NaCl, and 12.5% in the fraction eluted with 0.1 M NaCl. The CD63-positive fractions contained 83.8% of the recovered protein (i.e., 55.1% in the fraction eluted with 0.2 M NaCl and 28.6% in the fraction eluted with 1 M NaCl).

In agreement with this, the electrophoretic patterns indicated clear separation and/or enrichment of particular proteins in all five charge-resolved fractions (Figure 3D). Albumin (66 kDa band), which was used as a reference, was concentrated in the fraction eluted with 0.1 M NaCl, but was less abundant in the fraction eluted with 0.2 M NaCl and was strikingly reduced in the fractions eluted with 0.1 M NaCl. SDS-PAGE of the total protein in the 0.2 M NaCl eluate revealed numerous bands in a wide molecular mass range (30 kDa–<200 kDa), resembling the total protein pattern in the low-density sucrose gradient CD63-positive fractions. The total protein pattern in fractions eluted with 1 M NaCl was similar to that separated in mid-density SDG CD63-positive fractions.

The AF proteome is very complex, and various protein–protein or other types of interactions are possible. For example, albumin generally forms complexes with different proteins/glycoproteins, including IgG and mucins, depending on the source examined. The existence of such complexes could certainly influence the elution pattern from the ion-exchange column in terms of the distribution of particular proteins, giving wider than expected profiles based on their existing charge properties. Generally, protein complexes can be both separated as EV-associated or co-eluted with them.

Taken together, although two separated EV populations differ in charge, protein pattern, and size, it cannot be definitely confirmed at this stage that they are different with respect to type or origin.

Whereas SDG centrifugation purifies EVs according to flotation density, separation of two CD63-positive populations using IEC may also be partially related to the specificity of glycans present on their surfaces (i.e., the ligand density and steric availability of charged moieties). The composition of charge-resolved afEVs fractions was also examined by lectin blotting (Figure 4). In contrast to the solid-phase assay, where lectin binding involves surface glycans, the patterns revealed by lectin blotting should also include cargo proteins/glycoproteins in the EVs. Each of the pooled and concentrated charge-resolved fractions of the EVs gave a complex pattern of partially overlapping bands in a wide molecular mass range when subjected to Con A blotting, except for the 0.1 M NaCl eluate (Figure 4A). That fraction predominantly contains albumin, which is a non-glycosylated molecule.

Con A can bind different types of N-glycans, including high mannosylated glycans (which are not sialylated) and complex and hybrid glycans (which could be sialylated). A sialic acid moiety significantly contributes to the negative charge of a molecule, so afEVs were additionally tested for SNA reactivity (Figure 4B). SNA is a sialic acid–binding lectin, recognizing Siαx2,6Gal on N-glycans and is considered to be more selective than Con A (29). Although the number of SNA-reactive bands was lower than those reacting with Con A, their patterns were more differentiated. There were clear differences between the SNA binding patterns of the afEV populations eluted with 0.2 M NaCl and 1 M NaCl, as well as divergence in relation to the non-EV-containing fractions.

In addition, the general patterns of N-glycans revealed by Con A and SNA were compared with those of IgG, an abundant AF glycoprotein (Figure 3C). IgG was predominantly found in the unbound fraction (56.3%), while low IgG immunoreactivity was detected in the 0.05 M NaCl (21.6%) and 0.2 M NaCl (14.6%) eluates and even less in the fraction eluted with 1 M NaCl (4.8%).

Taked together, our findings indicated simultaneous removal of major soluble contaminants as seen in the differences in the distribution of total protein/albumin and total N-glycans/IgG between CD63/CD81/CD9-negative and CD63/CD81/CD9-positive fractions, as well as separation of the latter into two populations. This makes anion-exchange chromatography superior to SDG centrifugation for EV purification.

The results obtained here point to IEC as a simple and efficient method for the purification of EVs that can successfully replace SDG centrifugation. The necessity for monitoring glycans to detect fine differences in the composition of EVs was also demonstrated. Although evaluated using AF, IEC could be applied to other EV source material, given the general negative charge of EVs (28,30) (Lim, S.K. 2017. Method of purifying exosomes. Patent Application, www.google.com/patents/WO2012087241A1). However, the delineated conditions regarding the characteristics of the gradient (salt concentrations/volumes) and the choice of lectins for glycan analysis should be tested and adapted as needed. Glycoprotein composition is an important factor influencing the separation in general, and it can be tissue- and cell-specific or significantly change during growth and development. For instance, sialylation patterns in fetal tissues are different from those in adult tissues and also change during malignant transformation, depending on cancer type and progression (31).

Author contributions
M.K. and M.J. designed the study and wrote the manuscript, M.K., B.M., and M.J. interpreted the results, while M.K., B.M., S.G., and N.M. conducted the experiments.

Acknowledgments
This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, project No. 173010.

Competing interests
The authors declare no competing interests.

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Received 20 March 2017; accepted 22 June 2017.

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