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Vesicular uptake of macromolecules by human placental amniotic epithelial cells

Rita Sharshiner^a, Robert A Brace^{a,b}, and Cecilia Y Cheung^{a,b}

^aDepartment of Obstetrics and Gynecology, Division of Maternal Fetal Medicine, Oregon Health and Science University, Portland, Oregon USA

^bCenter for Developmental Health, Oregon Health and Science University, Portland, Oregon USA

Abstract

Introduction—Studies in animal models have shown that unidirectional vesicular transport of amniotic fluid across the amnion plays a primary role in regulating amniotic fluid volume. Our objective was to explore vesicle type, vesicular uptake and intracellular distribution of vesicles in human amnion cells using high- and super-resolution fluorescence microscopy.

Methods—Placental amnion was obtained at cesarean section and amnion cells were prepared and cultured. At 20%–50% confluence, the cells were incubated with fluorophore conjugated macromolecules for 1–30 minutes at 22°C or 37°C. Fluorophore labeled macromolecules were selected as markers of receptor-mediated caveolar and clathrin-coated vesicular uptake as well as non-specific endocytosis. After fluorophore treatment, the cells were fixed, imaged and vesicles counted using Imaris[®] software.

Results—Vesicular uptake displayed first order saturation kinetics with half saturation times averaging 1.3 minutes at 37°C compared to 4.9 minutes at 22°C, with non-specific endocytotic uptake being more rapid at both temperatures. There was extensive cell-to-cell variability in uptake rate. Under super-resolution microscopy, the pattern of intracellular spatial distribution was distinct for each macromolecule. Co-localization of fluorescently labeled macromolecules was very low at vesicular dimensions.

Conclusions—In human placental amnion cells, 1) vesicular uptake of macromolecules is rapid, consistent with the concept that vesicular transcytosis across the amnion plays a role in the regulation of amniotic fluid volume; 2) uptake is temperature dependent and variable among

Correspondence: Robert A. Brace, PhD Department of Obstetrics and Gynecology, Division of Maternal Fetal Medicine, L-458, Oregon Health and Science University, 3181 S.W. Sam Jackson Park Road, Portland, OR 97239-3098, Phone: 503-494-1235, bracer@ohsu.edu.

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Contributions

All authors contributed to the design, experimental data collection, data analysis, and preparation of the manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest

individual cells; 3) the unique intracellular distributions suggest distinct functions for each vesicle type; 4) non-receptor mediated vesicular uptake may be a primary vesicular uptake mechanism.

Keywords

amniotic fluid; amnion; endocytosis; fluorescence microscopy; vesicular transport

1. Introduction¹

Of the nearly 4 million births in the US each year, 5%–10% are affected by abnormal amniotic fluid volume (AFV) and, in many cases, the etiology is unknown [1, 2]. Both oligohydramnios and polyhydramnios are associated with adverse pregnancy outcomes, including preterm birth, low birth weight, abnormal fetal lie, placental abruption, postpartum hemorrhage and cesarean delivery [2–4]. Presently, there are no effective therapies for treating aberrant AFVs despite efforts to understand AFV regulation.

Experimental studies in sheep suggest that AFV is primarily regulated by intramembranous absorption, that is, the rapid transfer of amniotic water and solutes across the placental amnion into the fetal circulation [5–11]. This intramembranous transport is largely a unidirectional process and is poorly correlated with osmotic gradients, suggesting that vesicular transport across amnion cells is the primary physiologic intramembranous transport mechanism [11–16]. Further, intramembranous solute fluxes increase linearly with volume fluxes, showing that bulk vesicular transport is a major contributor to intramembranous solute absorption [5, 12, 13, 15].

Although electron microscopy studies of human amnion have shown the presence of large numbers of intracellular vesicles and that extracellular fluid uptake is an extremely active process [17], vesicle type and kinetic characteristics have not been established.

Two types of vesicles that may be involved with receptor-mediated fluid transport are caveolar and clathrin-coated vesicles [18–25]. Caveolae are involved in many biological processes including endocytosis and transcytosis and play a major role in transcellular albumin transport [18, 20, 26]. Clathrin-coated vesicles endocytose fluid, various proteins, essential nutrients and ions, playing a role in cell homeostasis as well as transcellular transport [27–29]. In addition, there are at least two types of non-receptor mediated vesicles that may be involved in uptake of fluids and macromolecules. These include micropinocytes and macropinocytes [30].

Our objectives were to use specific fluorophore-labeled macromolecules as markers of receptor mediated and non-receptor mediated vesicular uptake [18, 20, 26, 31–36] to 1) determine whether caveolar and/or clathrin-coated vesicles are present in human amnion cells, 2) analyze the kinetic characteristics of vesicle uptake and 3) determine the spatial distribution of vesicles within amnion cells. Placental amnion cells were used for the study

¹Abbreviations

AFV (amniotic fluid volume), BSA (bovine serum albumin), CTB (cholera toxin B subunit), Dex (70 kDa dextran), DIC (differential interference contrast), Tfn (transferrin)

as transport of amniotic fluid and solutes into fetal blood occurs across the placental rather than across the reflected amnion [5, 16].

2. Materials and methods

2.1. Study population

Participants in this study were pregnant women with a term singleton gestation (≥ 37 weeks) and normal AFVs undergoing elective cesarean delivery prior to the onset of labor at Oregon Health and Science University in Portland, Oregon. Normal AFV was defined as an amniotic fluid index of 5–25 cm and deepest vertical pocket of 2–8 cm by ultrasound assessment within 7 days of delivery date. Ultrasound assessments were performed by either trained nurses or physicians. Exclusion criteria included multiple gestation, non-English and non-Spanish speaking, age < 18 years, known fetal anomalies, labor (defined by documented cervical change and regular contractions on day of delivery), suspected chorioamnionitis, and ruptured fetal membranes.

Participants were recruited and informed consents signed at the time of admission for their scheduled cesarean deliveries. Chart abstraction was performed by trained study personnel after tissue collection to obtain maternal and infant characteristics. All data were de-identified after collection. Research protocols and study procedures were approved by the Institutional Review Board at Oregon Health and Science University.

Mean maternal age ($n = 7$) was 32.6 ± 5.5 (SD) years. Five subjects were Caucasian and 2 were Hispanic. Mean gestational age at time of amnion collection was $39^{4/7} \pm 0^{5/7}$ weeks. Median AFI was 9.5 (range 6.5–11.8) cm. Forty three percent of infants were female and the median infant weight was 3459 (range 2920–3890) g.

2.2. Amnion tissue collection

Placental amnion was obtained from 7 subjects immediately upon delivery of the placenta at the time of cesarean section. The placental amnion was isolated by separating it from the underlying chorionic plate and trimming circumferentially 1 cm from the edge of the placenta and medially at least 1 cm from the umbilical cord insertion.

2.3. Amnion cell preparation and culture

Amnion tissue was either used immediately for explant studies or for preparation of amnion cell cultures.

Amnion explants were used initially to determine whether cells in the amnion would take up fluorophore labeled macromolecules and subsequently to examine intercellular vesicular uptake variability by low-resolution imaging. Cultured amnion cells were used to study vesicular uptake kinetics, co-localization and intracellular distribution by high- and super-resolution imaging.

To prepare amnion cell cultures, the membranes were minced, digested with 0.31% trypsin and the dispersed cells were plated onto 4-chamber IbiTreat coverslips (Ibidi USA, Inc., Madison, WI) for high-resolution imaging and Ibidi glass 4-chambered coverslips for super-

resolution imaging. Cells were maintained in DMEM/F12 culture medium supplemented with 10% FBS (fetal bovine serum, Thermo Fisher Scientific, Waltham, MA) and 1x antibiotic/antimycotic at 37°C in a humidified atmosphere of air supplemented with 5% CO₂. At 20%–50% confluence, the cells were used for labeling and imaging studies.

2.4. Amnion explant staining for low-resolution imaging

Fresh amnion was dissected into 10×10 mm fragments and incubated at 37°C in DPBS (Dulbecco's phosphate-buffered saline supplemented with calcium and magnesium, Hyclone Laboratories, Inc., Logan, Utah). At 1 hour, the DPBS was replaced with 25 µg/ml BSA-555 (bovine serum albumin Alexa Fluor 555, Thermo Fisher Scientific) in DPBS for 60 minutes. The BSA-555 was reconstituted, diluted and mixed prior to treatment. Following treatment, the membranes were washed, fixed in cold freshly prepared 4% PFA (paraformaldehyde, Sigma-Aldrich) in DPBS and nuclear counter-stained with NucBlue fixed cell ready probe (DAPI, 4',6-diamidino-2-phenylindole, Thermo Fisher Scientific). The explants were mounted using Fluoromount G (Southern Biotech, Birmingham, AL) on Superfrost™ microscopy slides (Thermo Fisher Scientific) with #1.5 glass coverslips.

2.5. Amnion cell staining for high-and super-resolution imaging

Cultured cells were serum withdrawn for 1 hour and treated with the following fluorophore labeled macromolecules (Molecular Probes, Thermo Fisher Scientific) in DPBS: CTB-488 (15 µg/ml, cholera toxin subunit B, Alexa Fluor 488 conjugate), BSA-488 (25 µg/ml, bovine serum albumin, Alexa Fluor 488 conjugate), BSA-555 (25 µg/ml, bovine serum albumin, Alexa Fluor 555 conjugate), Tfn-555 (20 µg/ml, transferrin, Alexa Fluor 555 conjugate), Dex-594 (15 µg/ml, Dextran 70 kDa MW lysine fixable, Texas Red conjugate). Fluorophore labeled conjugates were diluted to desired concentrations, centrifuged to remove potential particulates, and vortex mixed prior to treatment. These labeled macromolecules were selected as specific makers: CTB for caveolar vesicles, Tfn for clathrin-coated vesicles, BSA for receptor-mediated vesicular uptake and Dex as a marker for non-specific endocytosis including micro- and macropinocytosis [18, 20, 26, 31–36].

For kinetic studies, cultured cells were single labeled with each fluorescent molecule for 1, 3, 10 or 30 minute(s). These incubation times were chosen because our preliminary studies showed that uptake saturation occurred in less than 30 minutes, as illustrated in the results below. After each treatment period, cells were fixed immediately in cold 4% PFA for 45 minutes, washed and cell nuclei stained with DAPI.

For co-localization experiments, the cells were dual-labeled with the fluorescent molecules for 30 minutes, fixed, washed, and the cell nuclei stained with DAPI.

2.6. Image acquisition

Amnion explants were viewed on a laser scanning confocal microscope with a 25X objective under oil immersion and images acquired using Zen software (LSM 710 Elyra PS1 Laser Scanning Confocal Microscope, Zeiss, USA).

For the vesicular uptake kinetic studies, cultured cells fluorescent labeled at each time point were viewed with a 60X objective under oil immersion. Three-dimensional (3D) z-stack images were acquired using a wide-field high-resolution microscope equipped with a Coolsnap ES2 HQ camera (Nikon, USA) and SoftWoRx™ 6.1.3 software (DeltaVision Core Widefield Deconvolution Microscopy System, IX71, Olympus, USA). Two individual cells with no contact with surrounding cells from 4 different quadrants (8 cells total) in each chamber were randomly identified by the differential interference contrast (DIC) channel and imaged using appropriate fluorescent filters. Optical sections of each cell were acquired in 0.2 μm slices in the z-plane and 1024 $\times$ 1024 pixels in the x-y planes for 3D image reconstruction. The voxel size was 107 $\times$ 107 $\times$ 200 nm. The fluorescently labeled BSA, CTB, Tfn and Dex were excited with a standard DeltaVision™ filter set for green fluorescent protein (Alexa Fluor 488), tetramethylrhodamine (Alexa Fluor 555), and 594 nm (Texas Red) wavelengths. The camera settings were optimized for the most positive image and applied to all images for consistency. Cells not exposed to fluorophore were imaged and processed similarly as negative controls (n = 185) to evaluate and correct for autofluorescence. Raw images of individual cells were deconvolved using an algorithm of 10 iterations. The resulting image stacks were computed by Fourier transformation to reconstruct images in 3D. After processing, image size averaged 240 megabytes per cell.

To determine intracellular distribution and co-localization of vesicles in cultured amnion cells, images were obtained on a super-resolution structured illumination microscopy system with a 63X objective under oil immersion using an Andor iXon Ultra 897 EMCCD camera (Elyra PS1 LSM 710 Laser Scanning Microscopy System, Zeiss, USA). Optical sections of each cell were acquired in 0.1 μm slices in the z-plane and 1004 $\times$ 1002 pixels in the x-y planes for 3D image reconstruction. Raw images were processed for structured illumination in Zen 2012 (Zeiss). Structural alignment of the spectra was performed prior to co-localization analysis. Each super-resolution image of an individual cell required approximately 3 hours for acquisition and processing.

2.7. Image quantification, data presentation and statistical analysis

Vesicle quantification for all cultured amnion cells was performed with Imaris image analysis software v.8.1.2 (Bitplane Inc, South Windsor, CT). High-resolution images were deconvolved and corrected for autofluorescence. Vesicles within each cell were defined using the Spots function. The number of spots was determined by varying the histogram quality threshold within each image to maximize the number of vesicles identified within the cell while no or minimal (1–2) spots appeared outside the cell. This same standard was applied to all images. The number of spots within each cell was then quantified for each fluorescent marker. For co-localization determinations using super-resolution images, distances of 0.2, 0.1 and 0.05 μm between spot centers were analyzed in individual cells. Spatial distribution of vesicles was determined by identifying the center of each vesicle at 0.01 μm resolution in each of the x, y and z directions. The numbers of vesicle centers for each marker within each 1 μm increment in each direction were counted.

Vesicular uptake followed first order saturation kinetics. In order to quantify kinetic characteristics, least squares regression was used to fit the number of vesicles (N) at 1, 3, 10, and 30 minute(s) to the Michaelis-Menten equation:

$$N = N_{\max} \times t / (t + K_m)$$

where t represents time in minutes and K_m the time of half saturation. When an individual macromolecule was tested more than once in cells from the same subject, the K_m values were averaged.

Data are expressed as median and range, mean $\pm$ SE or mean $\pm$ SD as indicated. Unpaired t-tests and one factor ANOVAs were used to compare half saturation times. The intracellular distributions of two macromolecules within single cells were compared with a Chi square contingency test. Statistically significant differences were defined by a P value < 0.05 .

3. Results

Amnion explants viewed under low resolution displayed uptake of BSA throughout the explant after 60 minutes of treatment. However, there was extensive heterogeneity in uptake among cells within amnion explants, with some cells heavily labeled while other cells showed minimal uptake (Figure 1). At high resolution, cultured cells displayed similar heterogeneity in uptake of BSA, CTB, Tfn and Dex at every time point for each fluorophore conjugated macromolecule (Figure 2).

Table 1 summarizes the half saturation time constants as a function of temperature for each macromolecule in individual amnion cells. At 37°C, differences among macromolecules were significant (ANOVA, $P = 0.040$), with CTB-488 and Dex-594 uptake occurring more rapidly than BSA-488. At 22°C the uptake of Tfn-555 was slower while Dex-594 was faster compared to that of BSA-488 (ANOVA $P = 0.0036$). At 37°C, uptake averaged 3.8 times that at 22°C and this difference was significant for all molecules combined at each temperature (t-test, $P = 0.0054$).

When viewed under super-resolution, vesicle distribution was not uniform within individual cells, in part due to the nucleus being void of vesicles. Further, it was visually apparent in some cells but not others that intracellular distributions of vesicles differed for each pair of macromolecules. Figure 3a illustrates an extreme of differences in intracellular vesicle distributions for 2 different macromolecules, BSA and Tfn, whereas Figure 3b shows a more typical pattern in which differences in distribution were not visually apparent for BSA conjugated to 2 different fluorophores. However, statistical analysis of intracellular vesicle location revealed significant differences in spatial distribution in each of the x, y and z planes for each pair of vesicles even when not visually apparent (Figure 4). Analysis of super-resolution images showed that co-localization of 2 different fluorophore-labeled macromolecules was a strong function of the distances between vesicles. For pairs of macromolecules at 50, 100 and 200 nm separation distances, co-localization averaged (mean $\pm$ SE) $0.32\% \pm 0.09\%$, $2.1\% \pm 0.6\%$, and $14.2\% \pm 4.5\%$, respectively (Figure 5). The

percent of vesicles that co-localized was not correlated with the number of vesicles within the cells ($P > 0.10$).

4. Discussion

We found extensive variability in macromolecule uptake by individual amnion cells. Similar variability occurred for all 5 fluorescent markers and at all time points {1, 3, 10, and 30 minute(s)}. This is consistent with a prior observation of variable horseradish peroxidase uptake at 60 minutes [17]. This heterogeneity potentially provides a mechanism for extensive increases in trans-amnion transport in response to volume challenges observed in animal studies [13, 15] in that, rather than or in addition to increasing vesicular transport across actively transporting cells, non-transporting cells may be recruited to participate in transport.

Another finding is that uptake of every macromolecule occurred rapidly with estimated half saturation times varying from < 1 minute to 2 minutes at 37°C . At 22°C , uptake was slowed by almost a factor of 4. This temperature-dependence in rate of uptake suggests that vesicular endocytosis is a metabolically driven, active and energy consuming transport process [12, 37]. Because vesicular uptake was very rapid, future uptake experiments in live amnion cells may benefit from the use of super-resolution fluorescence microscopy such as stochastic optical reconstruction microscopy (STORM) [38]. Such methods have the advantage of imaging uptake events in real time with improved accuracy and resolution necessary for precise characterization of the vesicular uptake process.

Intracellular distribution of fluorophore labeled vesicles was not uniform as would be expected due to intracellular organelle structures. This was reflected by the spikes on the distribution curves in Figure 4. In cells treated simultaneously with 2 macromolecules, differences in their intracellular distributions were evident statistically even if not visually apparent. This is not surprising if the macromolecules are taken up by distinct types of vesicles and transported/processed differently within the cell. However, the intracellular distributions of BSA-488 and BSA-555, although visually similar, were statistically different. This suggests that small differences in fluorophore size and conjugation site may alter the binding of BSA to its receptors within the vesicles or may alter the ability of individual vesicles to internalize the fluorophore conjugated protein.

An objective of this study was to identify the type of uptake vesicles. As CTB is suggested to be specific for caveolae [18, 26] while Tfn is known to bind to receptors in clathrin-coated vesicles [31, 32], our finding of rapid uptake for both macromolecules suggests that both vesicle types are active in amniotic epithelial cells and thus may participate in transcytosis of amniotic fluid. Co-localization studies have been used to study macromolecule interactions [39]. Since BSA uptake was shown to be receptor-mediated in caveolae [40], we expected to detect co-localization of BSA and CTB, a marker for caveolae. However, at separation distance of 50 nm, equivalent to diameter of caveolae, we were unable to detect significant co-localization of CTB and BSA, suggesting that co-localization studies may not be applicable for identifying macromolecule binding to specific vesicle types. Further, using the same macromolecule conjugated to 2 different fluorophores, as in the case of BSA, no

significant levels of co-localization within individual vesicles were detected. We speculate that steric factors may have prevented co-localization of multiple macromolecules in the same vesicle. A notable finding was that uptake of Dex, compared to CTB and Tfn, was substantially faster at both 37°C and 22°C, suggesting that the types of vesicles responsible for endocytosis and transcytosis in amnion may be primarily the non-caveolar and non-clathrin coated type. This finding was not anticipated and deserves further exploration. Alternatively, the rapid uptake of Dex may reflect non-specific uptake by all vesicle types combined rather than by a single vesicle type.

The present study has limitations. In the kinetic analyses, image resolution was 200 nm as limited by the resolving power of the deconvolution microscope. This limited the possibility of determining whether one or more macromolecules were within single vesicles. In our time course studies, this resolving power should not affect our conclusions because our super-resolution data showed that, at resolutions less than 200 nm, there was little co-localization of labeled macromolecules. A second limitation was that, with current image analysis software, vesicle number estimation is subjective rather than absolute. We attempted to minimize subjective bias by applying the same counting criteria to all cells.

In conclusion, this study suggests that vesicular uptake in human placental amnion cells is a rapid process that potentially participates in amniotic fluid transcytosis. Furthermore, our findings indicate that, although both caveolar and clathrin-coated vesicles are present, non-receptor mediated vesicular uptake may be a primary uptake mechanism.

These studies extend our knowledge of the physiology of amniotic fluid transport in human amnion cells in pregnancies with normal AFV. A critical question that remains is whether vesicular uptake is altered with or perhaps forms the basis of aberrant AFVs. Normal vesicular transport of amniotic fluid across the amnion may average 600–800 ml/day as shown in fetal sheep [12], suggesting that enhanced amnion cell uptake could lead to oligohydramnios while reductions may contribute to the formation of polyhydramnios in humans.

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Sharshiner manuscript highlights

- in placental amnion cells, vesicular uptake of fluorophore labeled macromolecules is very rapid
- non-receptor mediated vesicular uptake is more rapid than receptor mediated uptake
- vesicular uptake is almost 4 times more rapid at 37°C than at 22°C
- intracellular vesicle distributions differ for each pair of macromolecules
- at vesicular dimensions, macromolecular co-localization is low

Figure 1.

A low resolution image of a human amnion explant labeled with BSA-555 showing heterogeneity in cellular uptake of BSA after 60 minutes of fluorophore treatment. **a)** DIC (differential interference contrast, blue) image of amnion explant; **b)** uptake of BSA-555 (red) in the explant; **c)** a composite overlay image of DIC and BSA-555. Scale bar = 20 μm .

Figure 2.

Representative uptake kinetics at 22°C and 37°C as a function of time and macromolecule in cultured human amnion cells imaged at high resolution. Each dot represents the total number of labeled vesicles in a single cell for the respective macromolecule. Regression line and 95% confidence intervals are shown. R value is the correlation coefficient between the data and the regression line. K_m = half saturation time.

Figure 3.

a) A 2-dimensional (2D) maximum intensity projection of a 3D super-resolution image of a human amnion cell showing intracellular spatial distributions of BSA-488 (green) and Tfn-555 (red) after 30 minutes of fluorophore treatment. This example represents extreme differences in intracellular spatial distributions between 2 macromolecules. BSA was located mainly in the cell periphery while Tfn was found more centrally localized around the nucleus. **b)** A 2D maximum intensity projection of a 3D super-resolution image of a human amnion cell showing intracellular spatial distributions of BSA-488 (green) and BSA-555 (red) after 30 minutes of fluorophore treatment. This is an example of typical intracellular

spatial differences in distribution of vesicles labeled with the same macromolecule conjugated to 2 different fluorophores (shown) or 2 different molecules conjugated to 2 different fluorophores (not shown). Both BSA molecules were widely dispersed, with no visually apparent differences in distributions. For images in **a)** and **b)** each dot represents a vesicle 50 nm in diameter. Dot size is shown at 150 nm to enhance visibility. Scale bars as shown.

Figure 4.

Statistical analysis by Chi Square of the intracellular spatial distribution of vesicles in the X, Y, and Z planes of super-resolution images of **a)** BSA-488 and Tfn-555 and **b)** BSA-488 and BSA-555 of the cells in Figure 3a and 3b, respectively. Although statistically different distributions of the 2 phores in Figure 3a are not unexpected as they are visually apparent, the significant differences in distributions in Figure 3b were not anticipated because they appeared visually homogenous. Zero on each axis represents the edge of the image rather than edge of the cell. The value of df represents the degrees of freedom in the statistical test.

Figure 5.

Summary of co-localization measurements of pairs of fluorophore labeled macromolecules as a function of the distance between the centers of the vesicles after 30 minutes of fluorophore treatment. Data were obtained from 20 super-resolution images of single human amnion cells from 5 subjects and are presented as mean + SE. SE bar is within the dot if not visible.

Table 1

Half saturation time constants (K_m) as a function of temperature in individual human placental amnion cells.

	37°C	22°C
Macromolecule	mean ± SE (n)	mean ± SE (n)
BSA-488	1.80 ± 0.72 (3)	5.66 ± 2.17 (3)
BSA-555	1.20 ± 0.34 (5)	4.32 ± 0.96 (3)
CTB-488	0.55 ± 0.16 (3) *	3.09 ± 2.07 (3)
Tfn-555	1.99 ± 0.10 (3)	12.47 ± 1.07 (2) *
Dex-594	0.27 ± 0.18 (3) *	0.47 ± 0.26 (3) *
Overall	1.17 ± 0.22 (17)	4.40 ± 1.12 (15)

Data calculated assuming first order saturation kinetics from over 2000 high resolution images of single cells from 7 subjects. Alexa Fluor conjugated macromolecules: BSA-488 and BSA-555, bovine serum albumin; CTB-488, cholera toxin B subunit; Tfn-555, transferrin; Dex-594, 70kDa dextran. Comparison among individual macromolecules at each temperature was performed using a one factor ANOVA followed by post hoc testing with Fishers least significant difference for multiple comparisons. n is the number of subjects except n for Overall is the total of the above n values.

* $P < 0.05$ compared to BSA-488 at the same temperature.