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Prestin binding peptides as ligands for targeted polymersome mediated drug delivery to outer hair cells in the inner ear

Elena V. Surovtseva[a,*], Alexander H. Johnston[b], Weikai Zhang[a], Ya Zhang[a], Adele Kim[a], Michio Murakoshi[c], Hiroshi Wada[c], Tracey A. Newman[d], Jing Zou[a], Ilmari Pyykkö[a]

[a] Department of Otolaryngology, University of Tampere, Tampere 33014, Finland
[b] Centre for Biological Sciences, Life Sciences Building 85, Highfield Campus, University of Southampton, SO17 1BJ, United Kingdom
[c] Department of Bioengineering and Robotics, Tohoku University, 6-6-01 Aoba-yama, Sendai 980-8579, Japan
[d] Clinical Neurosciences, Life Sciences Building 85, Highfield Campus, University of Southampton, SO17 1BJ, United Kingdom

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ABSTRACT

Targeted delivery of treatment agents to the inner ear using nanoparticles is an advanced therapeutic approach to cure or alleviate hearing loss. Designed to target the outer hair cells of the cochlea, two 12-mer peptides (A_{665} and A_{666}) with affinity to prestin were identified following 3 rounds of sequential phage display. Two-round display with immobilized prestin protein was used to enrich the library for full-length prestin. The last round was performed using Cos-7 cells transiently transfected with a cCFP-prestin plasmid to display phages expressing peptides restrictive to the extracellular loops of prestin. The binding properties of A_{665} and A_{666} shown by flow cytometry demonstrated selectivity to prestin-expressing Chinese hamster ovary cells. PEG6K-*b*-PCL19K polymersomes covalently labelled with these peptides demonstrated effective targeting to outer hair cells in a rat cochlear explant study.

1. Introduction

Outer hair cell (OHC) dysfunction or loss from the cochlea is most common reason for hereditary or acquired hearing loss (Chen and Henderson, 2009; Clifford and Rogers, 2009; Fechter et al., 2007; Hodgkinson and Prasher, 2006; Leitner et al., 2011; Vrijens et al., 2008). Targeted drug delivery to the OHCs in the cochlea is an advanced therapeutic approach for treating sensorineural hearing loss associated with OHC pathology of a number of etiologies. This targeted approach to drug delivery relies on the selection of target molecules restricted to the specific cell population, in this case the OHCs. Prestin is a unique protein in the inner ear that is solely expressed in OHCs (Dallos and Fakler, 2002; Liberman et al., 2002; Zheng et al., 2000). It is an essential component of the membrane-based motor that enhances electromotility of OHCs and contributes to frequency sensitivity and selectivity in mammalian hearing. Therefore, prestin is a candidate molecule for targeting drug delivery to OHCs.

Prestin belongs to the SLC26 superfamily of anion-bicarbonate transporters which are highly homologous and show conservation across non-mammalian and mammalian species, including humans (Lohi et al., 2000). Two models of secondary structure have been proposed for prestin (Deak et al., 2005; Zheng et al., 2001). Subsequent reports have shown either 10 transmembrane helices (Navaratnam et al., 2005), or 12 transmembrane helices (Oliver et al., 2001) with 10 α-helices crossing the membrane and 2 non-membrane spanning helices (Deak et al., 2005). The extracellular loops of prestin may be suitable as binding motifs to enable the development of a cell-specific targeting strategy (Zheng et al., 2001). Due to their targetability towards specific cell types, biocompatibility and versatility in carrying payloads of different therapeutic agents, polymersomes (PMs) have potential as drug delivery vectors for the treatment of sensorineural hearing loss. PMs formed from polyethylene glycol-*block*-polycaprolactone (PEG-*b*-PCL) have been investigated as delivery vehicles of therapeutic agents to the inner ear (Anderson et al., 2008; Roy et al., 2010; Zhang et al., 2010). In *in vitro* cultures, the uptake of PMs into spiral ganglion cells (SGCs) was observed over periods of 24 h exposure with no indication of toxicity (Anderson et al., 2008). The targetability of functionalized PEG-*b*-PCL PMs to the SGCs, Schwann cells and nerve fibers was displayed in cochlear explants by conjugating a short peptide sequence that specifically binds to the tyrosine kinase

* Corresponding author.
E-mail addresses: Elena.Surovtseva@uta.fi, esurovtseva@gmail.com (E.V. Surovtseva).

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B (TrkB) receptor (Roy et al., 2010). PEG-*b*-PCL PMs with an affinity to prestin may be a suitable vector to mediate targeted drug delivery to OHCs. In this study, we identified two prestin-specific binding peptides (A_{665} and A_{666}) using phage display. Effective binding of the peptides to prestin was demonstrated in prestin expressing CHO-FLAG-prestin cells using flow cytometry. PEG-*b*-PCL PMs labelled with the A_{665} and A_{666} peptides demonstrated effective targeting to OHCs in a cochlear explant study.

2. Materials and methods

2.1. DNA construct and cell lines

The wild type Chinese hamster ovary (CHO-WT) cell line and CHO-FLAG-prestin cells which express FLAG-tagged gerbil prestin were prepared as reported previously (Murakoshi and Wada, 2009). The cCFP-prestin vector expressing fused Cerulean-prestin of mouse was kindly provided by Professor Jian Zuo (Department of Developmental Neurobiology, St. Jude Children's Research Hospital, Memphis, TN, USA). This construct was generated by replacing eGFP to Cerulean in the pEGFP-N1 (Clontech, USA) followed by cloning of a mouse prestin gene.

This construct was used to transfect Cos-7 cell line which was provided by Department of Cell Biology (University of Tampere Medical School, Tampere, Finland). Cos-7 cells expressing Cerulean-prestin were employed in the final biopanning in order to enrich for phages that express peptides that only bind to the extracellular part of prestin.

2.2. Cell culture and transient transfection

CHO-FLAG-prestin and CHO-WT cells were cultured using CHO-S-SFM II media (Gibco, USA) in humidified atmosphere at 37 °C.

Cos-7 cells were cultured in DMEM (Sigma–Aldrich, USA) supplemented with 10% FBS, they were grown in a humidified atmosphere at 37 °C and 5% CO_2.

Mouse prestin was transiently transfected into Cos-7 cells with a cCFP-prestin construct using a transfection reagent Lipofectamine (Invitrogen, USA). The transfection and membranous localization of Cerulean-prestin was evaluated by visual inspection of expression under fluorescent microscopy (excitation Filter 465–495 nm, barrier filter BA 515–555 nm), and at 48 h post-transfection, the cells were placed in media containing 250 μg/mL of G418. The transfected cells were used for phage display analysis after 24 h of incubation in the selection media.

2.3. Prestin protein purification

Prestin was isolated from CHO-FLAG-prestin cells by affinity chromatography using a Flag M purification kit (Sigma–Aldrich, USA), according to a previously published method (IIda et al., 2008). The purified FLAG-prestin was used in the first two rounds of biopanning to enrich phages expressing peptides.

2.4. Western blot analysis

Prestin solution was applied to a 15% SDS-PAGE gel, electrophoresed and transferred to a Hybond-P (Amersham, USA) nitrocellulose membrane. Non-specific binding sites were blocked with blocking reagent (Amersham, USA) in TBS containing 1% Tween 20. The blot was probed with goat anti-prestin IgG (Santa-Cruz Biotechnology, USA) at a dilution of 1:500. The primary antibody was visualized using horseradish peroxidase-coupled bovine anti-goat IgG (Santa-Cruz Biotechnology, USA) at a dilution of 1:10,000 followed by peroxidase-catalyzed chemiluminescence (Amersham, USA).

2.5. Phage display analysis

The first step in the sequential analysis was performed using prestin protein, which was attached to polystyrene dishes overnight at 4 °C with gentle shaking. A Ph.D.-12TM Phage Display Peptide Library Kit (New England Biolabs, USA) was used for phage selection. After washing twice with cold PBS, 10 μL of the initial phage library was added to the prestin-coated dish. The dishes were washed 10 times with cold, autoclaved TBS. Bound phages were eluted with a 0.2 M glycine buffer at pH 2.4 for 2 min followed by rapid adjustment of the pH to 8.0 and overnight treatment with PEG (MW = 8000, 1/6 (v/v)). The supernatant was tested for plaque formation and the phages were amplified. The second round of phage display was performed with the amplified phages.

The last round of phage display was performed with Cos-7 cells transfected with the cCFP-prestin plasmid to enrich the phages that bind extracellular loops of prestin. Cos-7 cells transfected with the cCFP-prestin plasmid were grown to ~80% confluence and washed with serum free media. 20 μL of the eluted phage library obtained after the second round was diluted in 5 mL of serum free media and applied onto cells transiently transfected with the cCFP-prestin plasmid. After 30 min of incubation at 10 °C to prevent phage internalization, cells were washed 10 times with cold, autoclaved TBS. Bound phages were eluted and subsequently amplified as above. Plaques from the 3rd eluate were collected and amplified individually followed by purification and sequencing of phage DNA. The final phages express peptides that have shown affinity to the extracellular loops of prestin. Sequencing was performed using the −96 sequence primer 5′-CCCTCATAGTTAGCGTAACG-3′. Sequences were registered by a commercial service provider (Oligomer Oy, Helsinki, Finland) and analyzed by Chromas. Selected peptides were synthesized using FMOC chemistry with N-terminal FITC conjugates on an automatic peptide synthesizer by Storkbio (Tallinn, Estonia). Peptides showing 95% purity by HPLC were further confirmed with MALDI-TOF mass spectrometry by a commercial service provider Storkbio (Tallinn, Estonia).

2.6. Flow cytometry

The transfection efficiency of Cos-7 cells with the cCFP-prestin plasmid and the binding of synthetic peptides to CHO-prestin cells were determined using flow cytometry with 488 nm excitation (Blue laser) and FL1: 530 ± 15 nm (FITC/GFP) (FACSAria®, BD Biosciences, Erembodegem, Belgium). For the binding measurements, CHO cells were incubated with A665 and A666 peptides at concentrations of 0.17 μg/mL, 0.5 μg/mL, and 5 μg/mL for 30 min before flow cytometry.

2.7. Polymersome synthesis

2.7.1. Preparation of peptide functionalized polymer

NH_2-PEG6K-*b*-PCL19K (60.0 mg) (Polymer Source Inc., Canada) was dissolved in dimethylformamide (DMF) (2.0 mL). 4-nitrophenyl-iodoacetate (10 mg) was added and the reaction mixture stirred for 4 h. Diethyl ether (50.0 mL) was added and the solution left overnight. The resulting precipitate was filtered and washed with diethyl ether to give iodoacetate-PEG6K-*b*-PCL19K (45 mg, 75% yield). Iodoacetate-PEG6K-PCL19K (15 mg) was dissolved into DMF (1 mL) and 1 mg of a cysteine-terminated peptide was added. The reaction mixture was stirred overnight, lyophilized and used crude for nanoparticle preparation. The peptide sequences used for the A_{665} peptide and A_{666} peptides were NH_2-Leu-Ser-Thr-His-Thr-Thr-Glu-Ser-Arg-Ser-Met-Val-COOH and

NH_2-Leu-Glu-Pro-Arg-Trp-Gly-Phe-Gly-Trp-Trp-Leu-Lis-COOH, respectively.

2.7.2. Polymersome preparation

The carboxy cyanine dye 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Invitrogen, UK) was dissolved in DMF at a concentration of 0.1 mg/mL. A_{665}-PEG6K-*b*-PCL19K (6.0 mg) was dissolved in 0.4 mL of the DMF/DiI solution. The polymer solution was then added dropwise (~1 drop every 8 s) to rapidly stirring PBS (1.60 mL). The sample was then dialyzed for 48 h against PBS with regular changes of the buffer solution. Analysis by dynamic light scattering showed the typical size of the particles to be 105.0 ± 20.0 nm. PMs labelled with the A_{666} peptide were made in an identical fashion using A_{666}-PEG6K-*b*-PCL19K and displayed similar sizes to the A_{665} labelled PMs. Unlabelled PEG-*b*-PCL control PMs were prepared by the same method (Zhang et al., 2010) but substituting methoxy terminated PEG5K-*b*-PCL5K for the peptide labelled polymer. Prior to use in cell culture experiments the PM samples were sterile filtered through a 0.2 μm cellulose acetate syringe filter and used immediately.

2.8. Cochlear explant experiment

All animal experiments were approved by the Ethics Committee of the University of Tampere. Animal care and experimental procedures were conducted in accordance with European legislation. P7–p10 newborn rats were decapitated after deep anaesthesia with Domitor (0.8 mg/kg medetomidine hydrochloride) and Ketalar (80 mg/kg ketemine hydrochloride) and sterilized with 70% ethanol. The cochleae were isolated and each cochlea was cut into 3–4 pieces and plated into a four-well Lab-Tek®II Chamber Slide (Nalge Nunc International) containing 1.0 mL defined medium/well (DMEM-F12 with B27 supplement, 1 mM n-acetyl-L-cystein, 1% penicillin–streptomycin, and 20 ng/mL epidermal growth factor (Sigma–Aldrich)). The cochlear explants were cultivated at 37 °C in a CO_2 incubator overnight and then treated with A_{665}-PEG6K-*b*-PCL19K PMs and A_{666}-PEG6K-*b*-PCL19K PMs at concentrations of 6 nmol/mL (0.15 mg/mL) and 3 nmol/mL (0.075 mg/mL) for 12 h. Unlabelled PEG5K-*b*-PCL5K PMs were added to the medium of cochlear explants at a concentration of 6 nmol/mL (0.06 mg/mL) and incubated for 20 h. At the end of the incubation, the cochlear explants were washed with phosphate-buffered saline 3 × 3 min and fixed in 4% paraformaldehyde for 30 min. After washing with PBS, a selection of the specimens were counterstained with FITC-labelled phalloidin 50 μg/mL (Sigma–Aldrich, USA) for 40 min to visualize the cytoskeletal F-actin, all the specimens were counterstained with DAPI 10 ng/mL for 10 min to demonstrate the nuclei, and mounted using Fluoromount for visualization by confocal microscopy, utilizing a Nikon inverted microscope Eclipse Ti installed with ANDOR IQ software. The excitation lasers were 488 nm, 568 nm and 405 nm from an Andor Laser Combiner system. The corresponding emission filters were 525/50 nm (FITC), 607/45 nm (TRITC) and 450–465 nm (DAPI).

3. Results

3.1. Characterization of isolated prestin protein

Prestin was purified from CHO-FLAG-prestin cells stably expressing prestin according to a previously published protocol (IIda et al., 2008). Prestin is a transmembrane protein containing a large hydrophobic core, consequently it requires detergent to purify and stabilize the soluble protein. Sucrose monolaurate using for this purpose shows a strong correlation between detergent concentration and solubilization efficacy. At 20 mM, this detergent has a solubilization efficacy of approximately 80%. Purification of prestin was confirmed by Western blot analysis using a monoclonal antibody against prestin (Fig. 1). Approximately 100 μg of prestin with a molecular weight of 80 kDa was obtained from 2×10^9 CHO-FLAG-prestin cells.

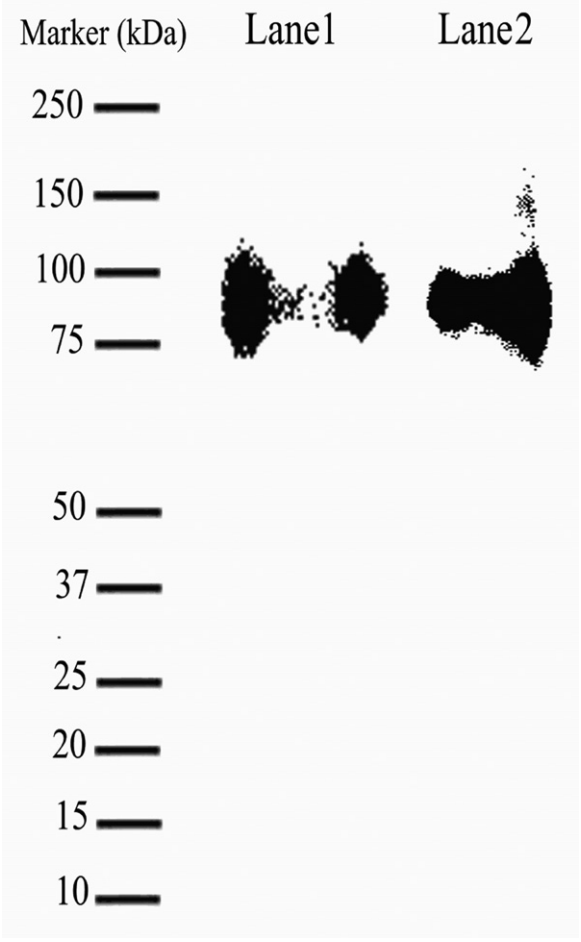

Fig. 1. The presence of prestin in CHO-FLAG-prestin cell lysates (1) and in elution from agarose-FLAG column (2). Western blot showed a band at 80 kDa of prestin that was resolved from CHO-FLAG-prestin cells with 20 mM sucrose monolaurate. The parent solution of monoclonal antibody against prestin was diluted 1:500.

3.2. Specific binding of phage display peptides to the extracellular loops of prestin

Prestin was adsorbed to polypropylene Petri dishes overnight at 4 °C, these were used to perform the first two of three rounds of biopanning. The 3rd round of biopanning was performed using Cos-7 cells transiently transfected with a cCFP-prestin plasmid, to eliminate false-positives affinitive to intracellular loops and transmembrane helices of the prestin that may have been obtained using soluble prestin. In this case only the library which expresses peptides with affinity to the extracellular loops of prestin was used to display final products all others were eradicated from resulting phage pattern. The biopanning experiments performed using Cos-7 cells were 40% positive for prestin expression as shown by flow cytometry. The membranous localization of the Cerulean-prestin fusion protein was observed under fluorescent microscope (Fig. 2).

Two prestin binding sequences were identified following sequencing of approximately 70 phage plaques that were analyzed

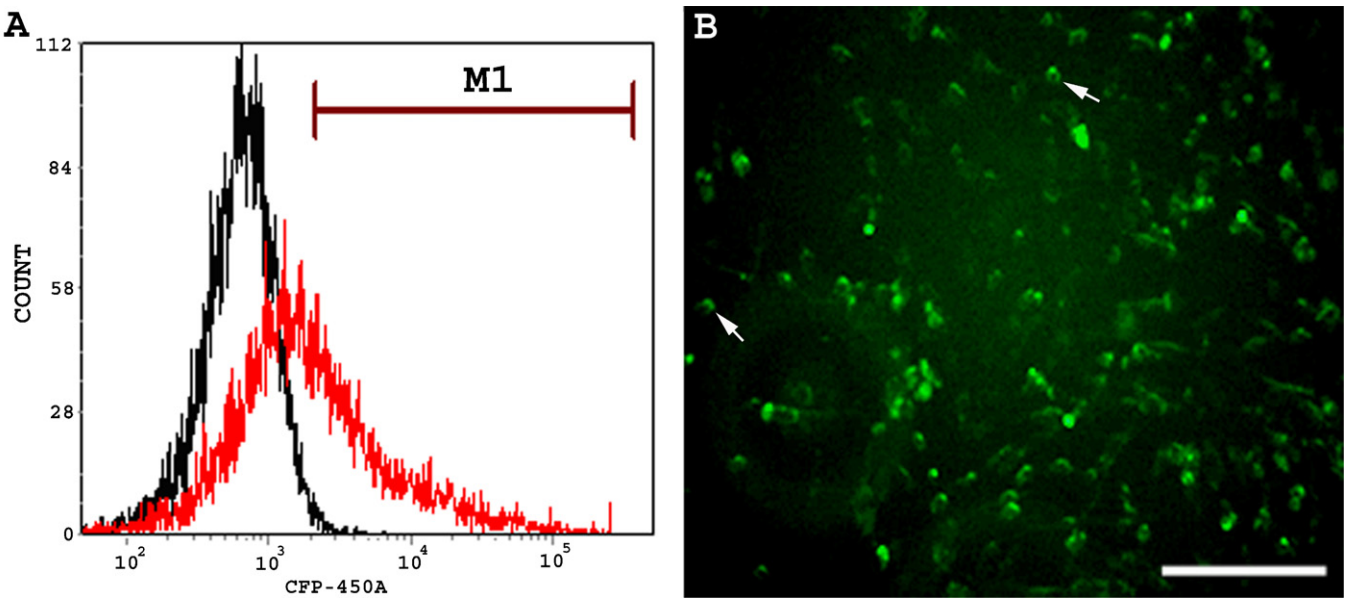

Fig. 2. Expression of prestin in the Cos-7 cells was confirmed by flow cytometry and fluorescent microscopy. Significantly higher percentage of M1 gated cells (38.99%, arithmetic mean fluorescence value = 11,580.5), which represent Cerulean-prestin expressing cells, in the transfected population than that of non-transfected Cos-7 cells (0.74%, arithmetic mean fluorescence value = 2751.51) (black curve) was obtained by flow cytometry (A). The membranous location of Cerulean-prestin in the cCFP-prestin plasmid-transfected Cos-7 cells was confirmed by fluorescent microscopy (arrows in B).

following 3 rounds of biopanning, NH_2-Leu-Ser-Thr-His-Thr-Thr-Glu-Ser-Arg-Ser-Met-Val-COOH (A_{665}) and NH_2-Leu-Glu-Pro-Arg-Trp-Gly-Phe-Gly-Trp-Trp-Leu-Lis-COOH (A_{666}).

The binding capacity of the FITC-labelled A_{665} and A_{666} peptides was confirmed by flow cytometry at concentrations of 5, 0.5 and 0.17 μg/mL within 30 min of incubation of CHO-FLAG-prestin or control cells with these peptides. The binding capacity of these peptides to CHO-FLAG-prestin cells was significantly higher than to CHO-WT cells that were used as a negative control (Fig. 3).

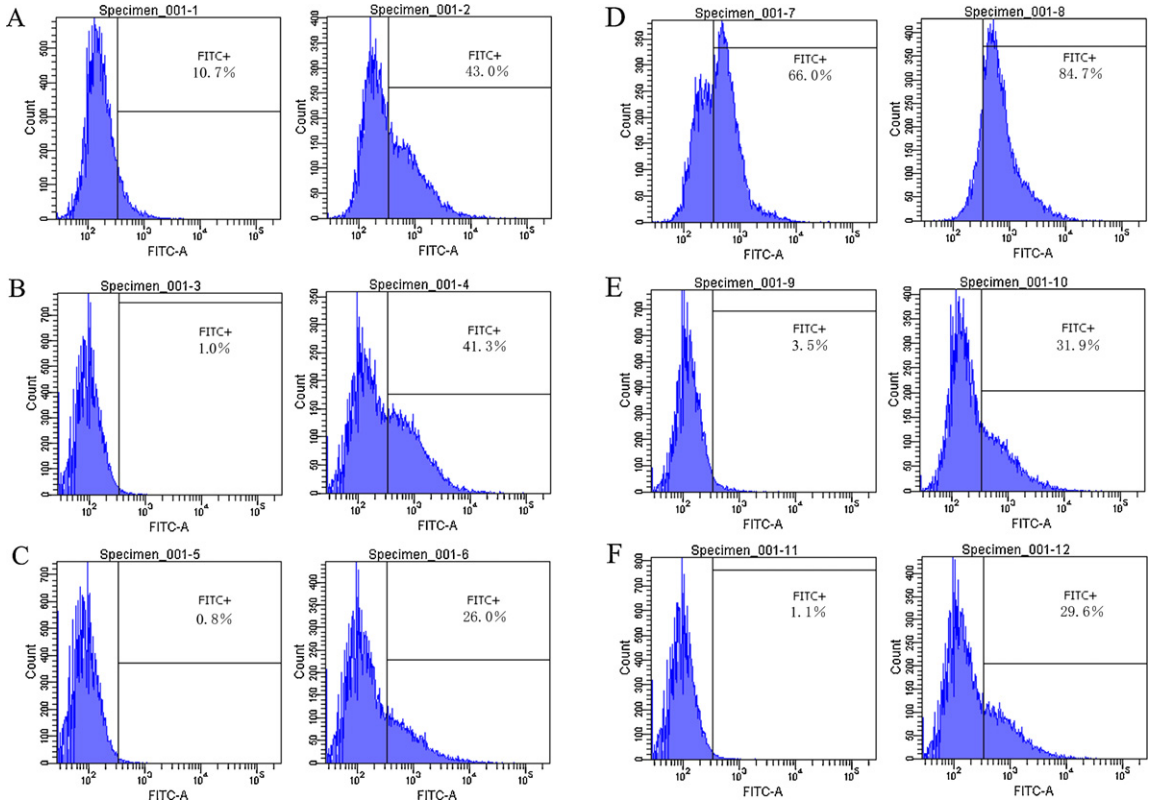

Fig. 3. Affinity and specificity of FITC-labelled A_{665} and A_{666} peptides to CHO-FLAG-prestin cells were demonstrated by flow cytometry. Both the A_{665} (A–C) and A_{666} (D–F) peptides showed specific affinity to the CHO-FLAG-prestin cells (CHO-prestin) (right bar) but not to the control CHO cells (CHO-WT) (left bar) at the concentrations of 0.5 μg/mL (B and E) and 0.17 μg/mL (C and F). However, non-specific binding to the control CHO cells was observed at the higher concentration of peptides 5 μg/mL (A and D).

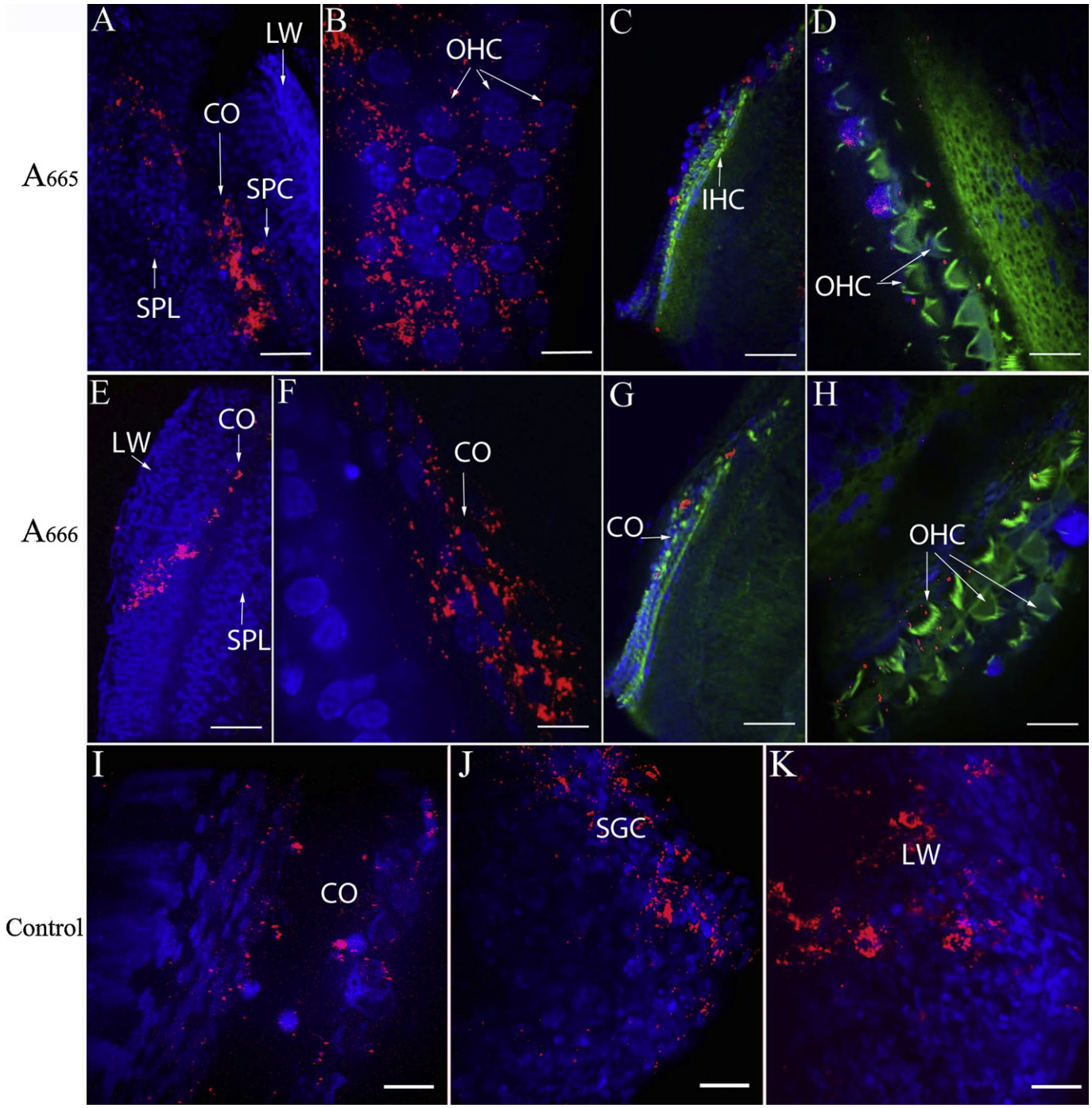

Fig. 4. Targeting to the outer hair cells (OHCs) by PEG-*b*-PCL PMs functionalized with A_{665} and A_{666} peptides was demonstrated using rat cochlear explants. After 12 h at a polymer concentration of 6 nmol/mL, both A_{665}-PEG6K-*b*-PCL19K (A and B) and A_{666}-PEG6K-*b*-PCL19K PMs (E and F) were detected abundantly in the region of OHCs, though appeared sparsely in the regions of supporting cells (SPC) and spiral limbus (SPL). More specific binding of both A_{665}-PEG6K-*b*-PCL19K (C and D) and A_{666}-PEG6K-*b*-PCL19K PMs (G and H) was demonstrated after incubation of 12 h at a lower concentration (3 nmol/mL), while random distribution of the unlabelled PEG5K-*b*-PCL5K PMs was shown in the Corti's organ (I), spiral ganglion cells (J), and lateral wall (K) when applied to the explant at a similar polymer concentration (6 nmol/mL). Red: PMs. Blue: contrast staining of nuclei with DAPI. Green: F-actin stained with FITC-conjugated phalloidin. Scale bars are 100 μm in A, C, E, and G; 10 μm in B, D, F, and H and I; 25 μm in J and K. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

At high peptide concentrations (5 μg/mL) significant non-specific binding to CHO-WT cells was observed (Fig. 3). However, even at these high concentrations, the binding of the A_{665} and A_{666} peptides to CHO-FLAG-prestin cells was significantly higher than to CHO-WT control cells. At lower peptide concentrations (0.5 and 0.17 μg/mL), the binding capacity of these peptides to CHO-WT cells was below 1% compared to ~30–40% for CHO-FLAG-prestin cells (Fig. 3). These data demonstrate that the A_{665} and A_{666} peptides result in specific binding to the extracellular loops of prestin.

3.3. *Targeting of OHCs using PEG-b-PCL PMs functionalized with the A_{665} and A_{666} peptides*

The selectivity to OHCs of PEG-*b*-PCL PMs functionalized with the A_{665} and A_{666} peptides was confirmed on the rat explants.

Following 12 h of incubation, the confocal images illustrate that A_{665}-PEG6K-*b*-PCL19K and A_{666}-PEG6K-*b*-PCL19K polymersomes (polymer concentrations of 6 nmol/mL and 3 nmol/mL) were abundantly internalized by the OHCs and only sparsely taken up by other cell types (Fig. 4). Some nonspecific binding was found at the high (6 nmol/mL) PM polymer concentration (Fig. 4). Low magnification images of samples incubated with low concentrations of A_{665}-PEG6K-*b*-PCL19K and A_{666}-PEG6K-*b*-PCL19K PMs revealed signal in the OHCs of the rat cochlea demonstrating targeting. In contrast, we observed non-specific internalization of unlabelled PEG5K-*b*-PCL5K PMs (polymer concentration 6 nmol/mL) by different cell types in the cochlear explants, including supporting cells and cells in the lateral wall.

4. Discussion

In this study, two peptides, designated as A_{665} and A_{666}, identified in a phage display library were developed and demonstrated to show specific binding to the prestin expressed on the surface of CHO-FLAG-prestin cells *in vitro*. The specificity of the peptides was established through multiple biopanning steps. In the first two rounds of biopanning, the phage display library was enriched to express peptides with affinity to the whole prestin protein. Finally, a library expressing peptides binding to the extracellular loops of the prestin was displayed using Cos-7 cells transfected by cCFP-prestin plasmid. The potential application of the two novel peptides in advanced nanomedicine was tested using a cochlear explant culture by conjugating these peptides to the surface of PEG-*b*-PCL PMs. These showed targeting to the OHCs of the rat cochlea in a concentration dependant manner. High concentrations of the functionalized PMs compromised the specificity of the PMs to the OHCs of the rat cochlea. Prior to use in clinical trials, an *in vivo* study needs to be performed to establish the optimal concentration of the PMs required to maintain targeting while also being at an adequate concentration to establish a sufficient level of drug release.

The plasmid in CHO-FLAG-prestin cells used in the first two rounds of biopanning to enrich phages expressing peptides was constructed with gerbil cDNA. The plasmid in Cos-7 cells expressing Cerulean-prestin, which were employed in the final biopanning to enrich phages that express peptides only bind to the extracellular part of prestin, was constructed with mouse cDNA. The rationale for choosing prestin cDNA from these two species is that they are available and 99% identical in the amino acid sequences. The 1% discrepancy between sequences falls within the intracellular domain, which does not influence the peptide binding activity (Currall et al., 2011). These do not hamper the future application in the clinic because the 3% difference in sequences between gerbil and human prestin also falls in the intracellular domain.

OHCs may be lost as a consequence of exposure to loud noise and other insults. However, up-regulation of prestin occurs in the remaining intact OHCs during noise exposure (Mazurek et al., 2007). It has also been reported that, consistent with an increase in the distortion product of otoacoustic emission, long-term administration of salicylate can increase prestin expression and OHC electromotility (Yu et al., 2008). In contrast prestin was down-regulated in mouse OHCs prior to detectable apoptosis and hearing loss after subcutaneous injection of kanamycin (Yu et al., 2011). These studies indicate that prestin itself is a new treatment target. Development of prestin specific peptides and the functionalized PMs has opened a window and provided a new opportunity to explore this issue. The consequence of prestin-conjugated PM administration on the prestin signaling, electromotility, and viability of OHCs needs to be investigated.

5. Conclusion

Sequential phage display identified two peptides (A_{665} and A_{666}) that bind selectively to the extracellular loops of prestin, which is a unique protein expressed only in OHCs of the cochlea. A potential application of the novel peptides in nanomedicine was demonstrated by showing targetability of polymersome nanoparticles to OHCs of the rat cochlea in an explant study. Development of these novel prestin specific peptides presents a new opportunity to explore the function of prestin in cochlear OHCs and the development of novel treatment strategies for sensorineural hearing loss.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

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