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Supplemental Information for:

Salinity increases the toxicity of silver nanocolloids to Japanese medaka embryos

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Silver nanocolloids

Purified SNCs (20 mg/L, 99.99% purity, particle mean diameter about 28.4 ± 8.5 nm suspended in distilled water) were purchased from Utopia Silver Supplements (Utopia, TX, USA). Diluted SNC solutions (a mixture of silver colloids and Ag^+) for exposure tests were prepared in each concentration of ERM (1×, 5×, 10×, 15×, 20×, or 30×) (1× ERM consisted of 1.0 g NaCl, 0.03 g KCl, 0.04 g $CaCl_2 \cdot 2H_2O$, and 0.163 g $MgSO_4 \cdot 7H_2O$ in 1 L of ultrapure water; pH adjusted to 7.2 with 1.25% $NaHCO_3$ in ultrapure water) and stirred overnight. All of the ERM reagents described were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). The purity and concentration of the silver were validated by ICP-MS analyses using a Thermo Scientific X Series 2 (Thermo Scientific, Pittsburgh, PA, USA). $AgNO_3$ was purchased from Nacalai Tesque, Inc. and used as a reference compound for SNCs.

Medaka eggs

The medaka (*Oryzias latipes*) orange-red strain was obtained from the National Institute for Environmental Studies, Tsukuba, Ibaraki, Japan. Breeding groups of medaka

were fed *Artemia salina* nauplii once a day and Otohime larval β-1 (Marubeni Nissin Feed Co. Ltd, Tokyo, Japan) five times a day and maintained under a 16:8-h light:dark cycle at 24 ± 0.5 ºC. After the female medaka had spawned, the external egg clusters were removed. Fertilized eggs were then selected, placed in 1× ERM, and incubated at 25 ± 0.1 °C in an incubator (TG-180-5LB, NKsystem, Osaka, Japan). Embryos at developmental stage 21 (brain regionalisation and otic vesicle formation stage) were harvested, rinsed with 1× ERM, and then used for the exposure experiments. The developmental stages of medaka embryos were defined by Iwamatsu in 2004.[1] All medaka embryos used were at stage 21, because our previous study had revealed that stage 21 was more sensitive than other stages to SNCs.[2]

Wave scanning of silver solutions

For qualitative analysis of SNC or $AgNO_3$ solutions or Ag-complex formation, SNC or $AgNO_3$ solutions (in 1×, 5×, 10×, 15×, 20×, or 30× ERM, or in ultrapure water) were wave-scanned with a UV-Vis-NIR spectrophotometer (UV-3600, Shimadzu Co., Kyoto, Japan; scan speed medium, sampling pitch 0.5 or 2.0 nm, slit width 2.0 nm, time constant 0.1 s) by using a quartz cell (optical path length 10 mm; model 200-34442,

Shimadzu GLC Ltd, Tokyo, Japan). Portions of the SNC or $AgNO_3$ solutions were filtered through a 3-kDa membrane filter (0.5-mL centrifugal-type filter, EMD Millipore Corporation, Billerica, MA, USA) at 14,000*g* and 4 ºC for 10 min; this filter size was chosen because the mean diameter of the aggregated SNCs in 1× ERM was 67.8 nm and that of Ag^+ was 0.162 nm,[2] and the 3-kDa membrane excluded particles of 2 nm or more.[3] We then measured the absorbances of the unfiltered and filtered solutions.

Salinity-dependent production of silver chloro complexes

Formation of silver chloro complexes was calculated by using Visual MINTEQ version 3.0 and stepwise formation constants for Cl^- and Ag^+.[4] To calculate silver chloro complex production we used Ag concentrations detected as soluble silver in 1×, 5×, 10×, 15×, 20×, or 30× ERM. The total ion concentration in 1× ERM (including chloride ions) was 18.447 mM, whereas the concentration of chloride ions alone was 18.058 mM. Chloride ions were therefore dominant in 1× ERM, accounting for *ca.* 97.9% of the total ion concentration. Therefore, for our calculations using stepwise formation constants, we used only the silver ion and chloride ion concentrations.

Measurement of silver uptake by medaka embryos and of soluble silver

To investigate the effects of osmotic pressure on silver uptake, 15 medaka eggs (stage 21) in triplicate were exposed to SNCs at 10 mg/L in each concentration of ERM (1×, 5×, 10×, 15×, 20×, or 30×) at pH 7 and 25 ºC in the dark for 5 days. Test solutions were renewed once a day. The concentration of Ag^+ in each test solution (50 µL) was analysed by using ICP-MS (see below). After exposure (day 6), three exposed eggs were dechorionated with medaka hatching enzyme, and the silver concentrations in the three embryos were then measured by ICP-MS. Details of the medaka-hatching enzyme are given in our previous paper.[5] All samples for ICP-MS were kept at –80 ºC until analysis. To isolate Ag^+ from each SNC solution (a mixture of silver colloids and Ag^+), 0.5 mL of test solution was filtered through a 3-kDa membrane filter (0.5-mL centrifugal-type filter, EMD Millipore Corporation) at 14,000*g* and 4 ºC for 10 min; this filter size was chosen for the same reasons as given above. The Ag^+ concentration in the filtered solution was measured by ICP-MS.

ICP-MS analyses of test water and medaka embryos

Two millilitres of ultrapure nitric acid (Ultrapur-100, specific gravity 1.42, Kanto Chemical

Co., Tokyo, Japan) was added to 50-μL water samples or embryo samples in a 50-ml Teflon beaker (Sanplatec Co., Osaka, Japan). The mixture was heated at 110 ºC until just before it dried out. Then 2.0 mL of ultrapure nitric acid and 0.5 mL of hydrogen peroxide (for atomic absorption spectrometry, Kanto Chemical Co.) were added to the beaker, which was again heated until just before the mixture dried out. The residue was dissolved in 1.0% ultrapure nitric acid solution to a volume of 12.0 mL and then subjected to ICP-MS analysis. Detection limits were 0.0018 ng/mL (water) and 0.016 ng/mg-weight (embryo body).

In our previous paper,[5] we measured the silver concentration in 100 eggs by using inductively-coupled plasma optical emission spectrometry (ICP-OES). In this study, we measured the silver concentration in only 3 dechorioned eggs, and we used ICP-MS. The detection limit of ICP-OES was 0.3 μg/L, whereas that of ICP-MS was 1.8 ng/L. ICP-MS is 170 times more sensitive than ICP-OES.

Measurement of electrical resistance

To examine the mechanism of salinity-dependent ion permeation, we measured the electrical resistance of the egg chorion in each concentration of ERM (1×, 5×, 10×, 15×,

20×, or 30×) at pH 7 and 25 ºC. For this purpose, we designed and assembled a system that included a regulated DC power supply (GMO8-10, Takasago Ltd, Kanagawa, Japan), an ammeter (YEW 1971 No. M1K 2082, Yokogawa Electric Works Ltd, Musashino, Tokyo, Japan), and a 0.8-mm Ag-AgCl electrode wire (E255, Warner Instruments, Hamden, CT, USA) (Figs. S6a and S6b). A single egg (stage 21) that had been incubated in one of the concentrations of ERM (1×, 5×, 10×, 15×, 20×, or 30×) at pH 7 and 25 ºC in the dark for 24 h was placed in the middle of a Teflon tube (length 15 mm, i.d. 1.07 mm, o.d. 1.67 mm; Chukoh Chemical Industries, Ltd, Minatoku, Tokyo, Japan) (Figs. S6a and S6b); both ends of the tube were then filled with the same ERM that had been used for the 24-h incubation. The electrical resistance of the egg (ρ) was calculated by using Formula S1, which was modified from a formula used for electrical resistance in electrical resistivity logging,[6] by using R, π, r, and L (see Formula S1 for definitions of these terms). The diameters of eggs that had been incubated in each concentration of ERM were also measured under a dissecting microscope with a micrometer before the electrical testing (Table S4). Resistance (R) was calculated by using the measured electric current and different constant voltages (0.4, 0.8, 1.2, 1.6, or 2.0 V) (see Figs. 4a and 4b).

Measurement of osmotic pressure

Two hundred medaka embryos (stage 21) were incubated in each concentration of ERM (1×, 5×, 10×, 15×, 20×, or 30×) at pH 7 and 25 ºC in the dark for 24 h. After the incubation, the solution around the eggs was removed with a paper towel and the eggs were crushed with a disposable hand-held homogenizer (BioMasher II, Nippi Incorporated, Tokyo, Japan). Homogenized solutions (embryonic fluids) were centrifuged at 100*g* and 4 ºC for 1 min to remove the egg chorion, and 20 μL of embryonic fluids was then subjected to triplicate measurement of osmotic pressure with an osmometer (Fiske One-Ten, Advanced Instruments, Inc., Norwood, MA, USA) (Fig. S7a). The osmotic pressure of each concentration of ERM was also measured and showed a linear relationship to the osmotic pressure of the embryonic fluids (Fig. S7b).

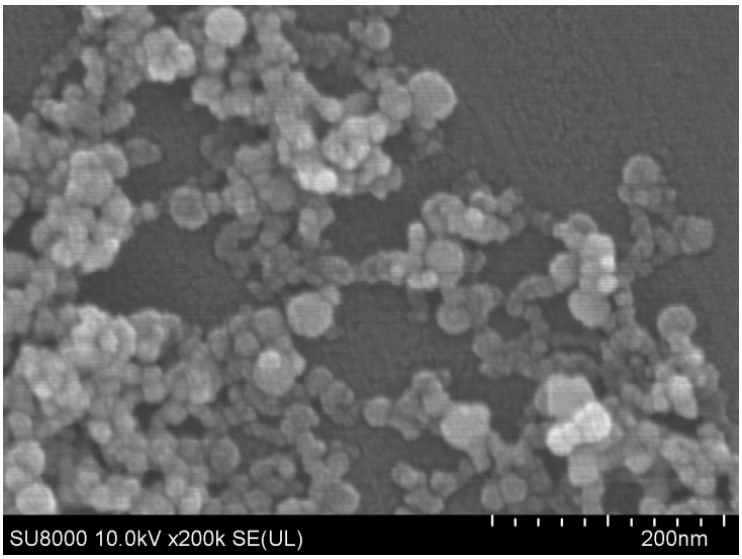

Figure S1. Scanning electron microscope (SEM) image obtained of silver nanocolloids (SNCs) in ultrapure water by using an S4800 SEM (JEOL, Tokyo, Japan) at 1.0 kV. The diameter of SNCs in ultrapure water was about 28.4 ± 8.5 nm.

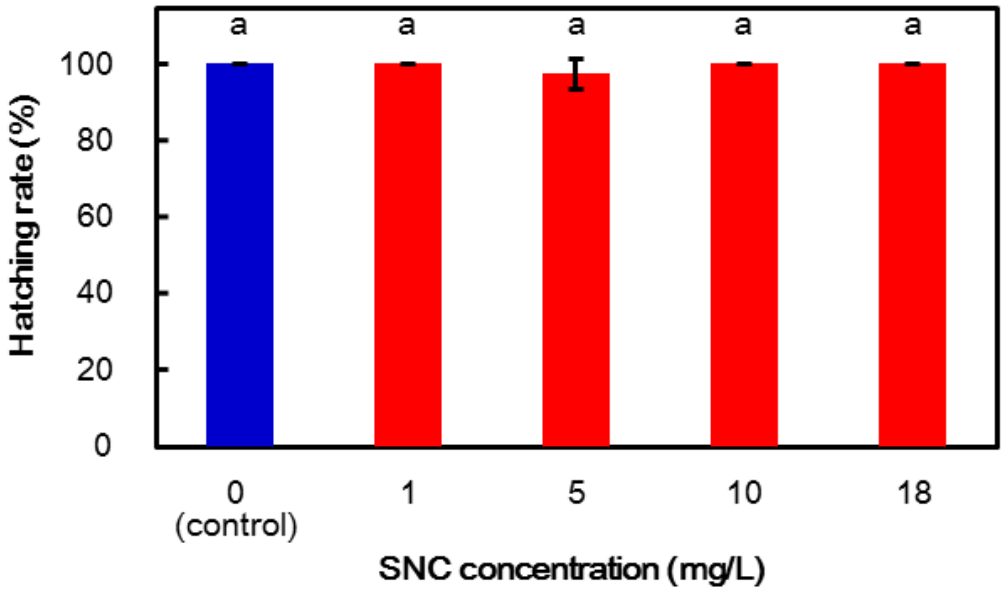

Figure S2. Hatching rates of medaka eggs exposed to silver nanocolloids (SNCs) in 1× embryo rearing medium (freshwater conditions). Cumulative hatching rates were counted under a dissecting microscope for 14 days.

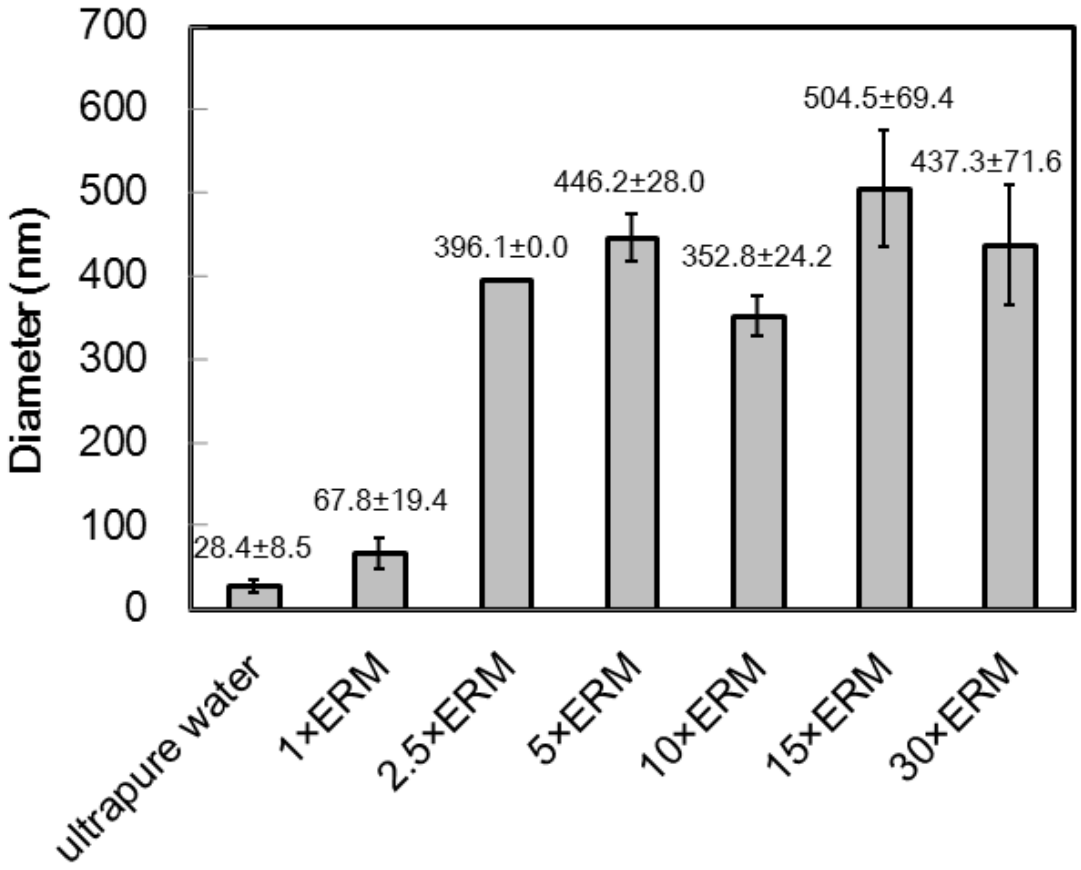

Figure S3. Diameters of aggregated silver nanocolloids in different concentrations of embryo rearing medium (ERM).

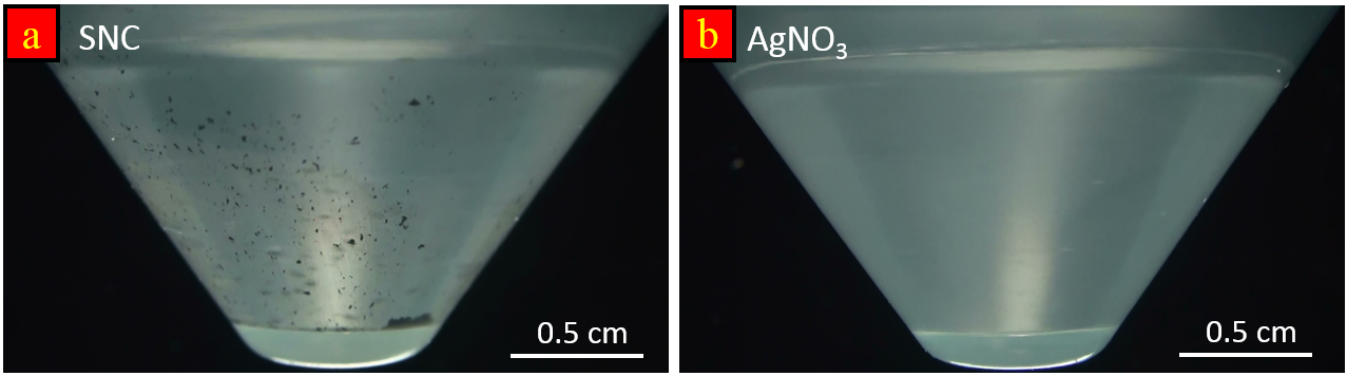

Figure S4. Comparative precipitation of silver nanocolloids (SNC) solution and silver nitrate solution ($AgNO_3$) in 30× embryo rearing medium (ERM). Black precipitates, probable aggregated SNCs were formed in SNC solution (a). There was no precipitate formed in $AgNO_3$ solution (b).

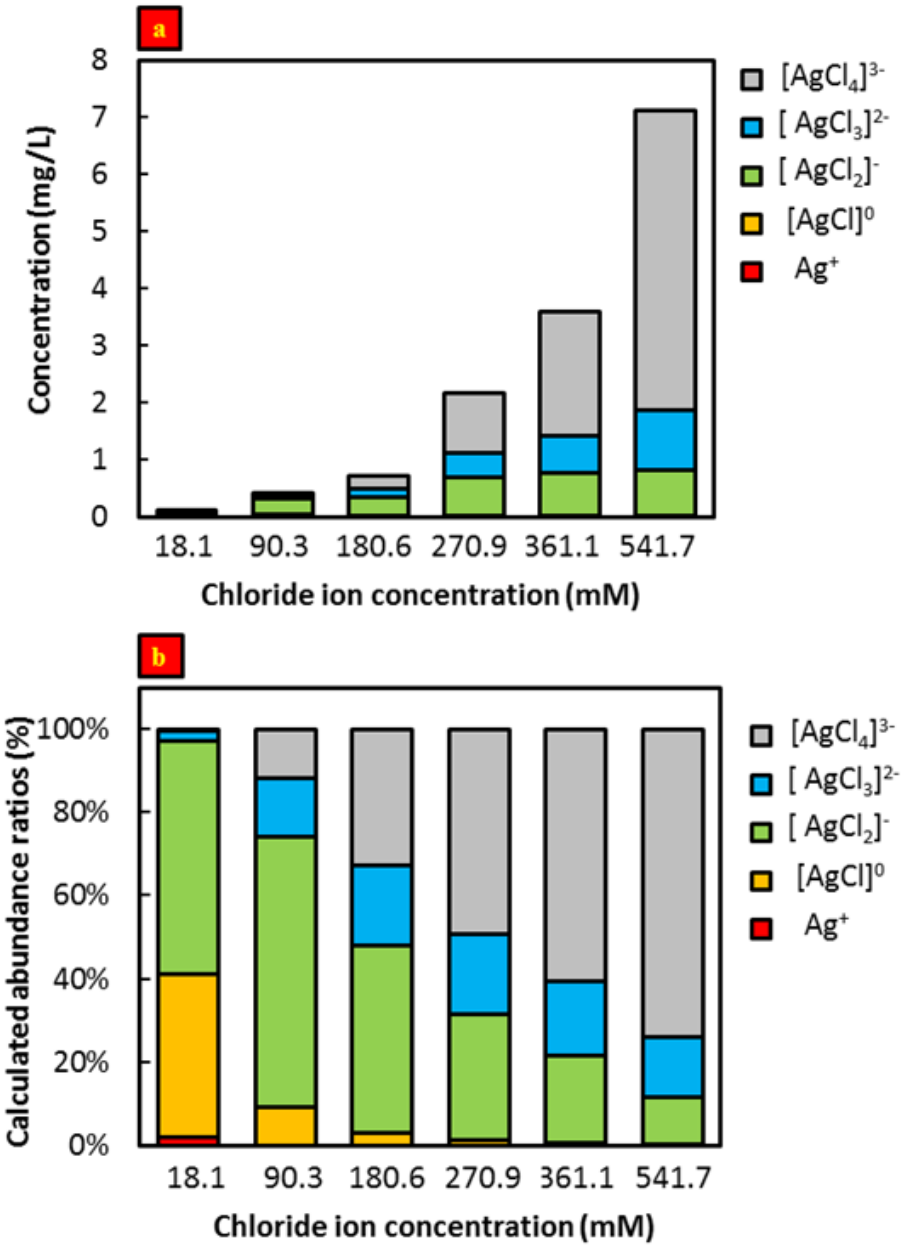

Figure S5 **Soluble silver concentrations calculated by using stepwise formation constants for Cl^- and Ag^+.**

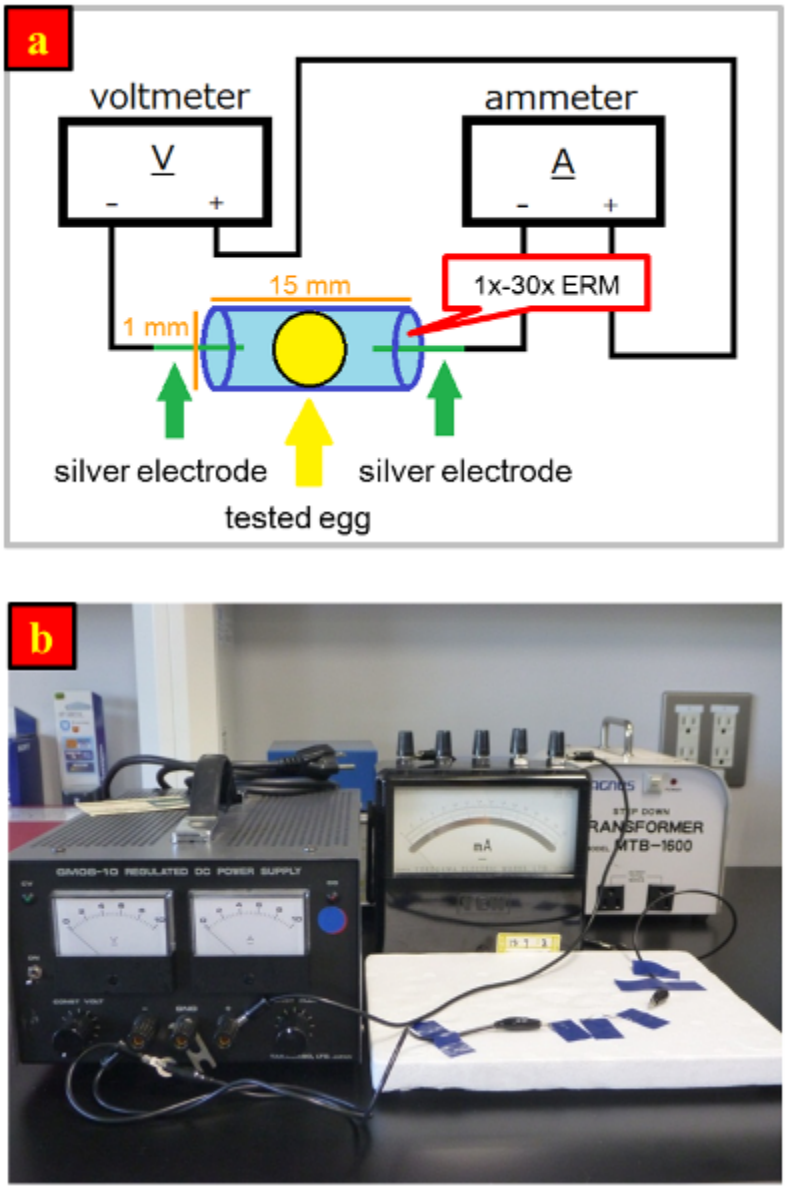

Figure S6. Measurement of electrical resistance in medaka egg chorion. (a) Schematic diagram of the device used to measure electrical resistance in the medaka egg chorion. (b) The measurement device.

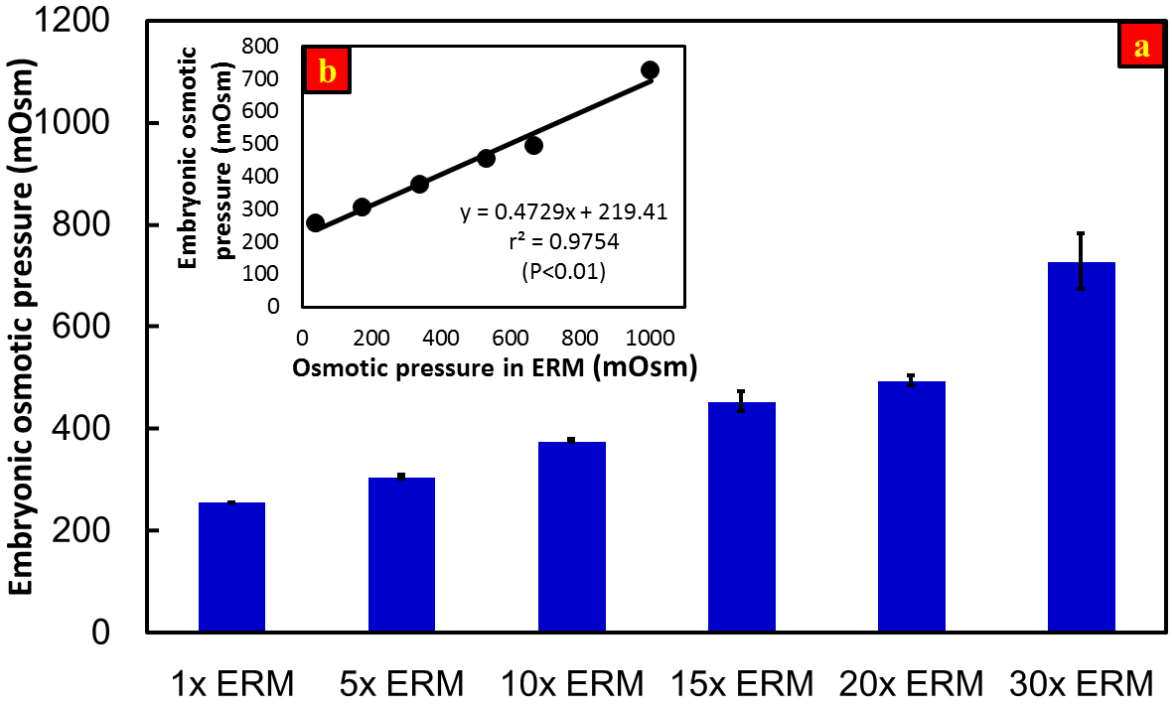

Figure S7. Osmotic pressures of body fluids from medaka embryos incubated with different concentrations of embryo rearing medium (ERM). (a) Osmotic pressures of body fluids from medaka embryos. (b) Relationship between osmotic pressure of body fluids from medaka embryos and each concentration of ERM.

Table S1. Percentages of chemical species in silver nanocolloid solutions, as calculated by using Visual MINTEQ version 3.0.

	1× ERM	5× ERM	10× ERM	15× ERM	20× ERM	30× ERM
Ag^+	0.9%	0.1%	0.0%	0.0%	0.0%	0.0%
$[AgCl]^0$	33.0%	8.2%	3.7%	2.2%	1.5%	0.9%
$[AgCl_2]^-$	64.5%	79.6%	72.5%	65.2%	59.2%	50.9%
$[AgCl_3]^{2-}$	1.6%	12.2%	23.8%	32.6%	39.2%	48.3%
AgOH	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
$[Ag(OH)_2]^-$	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
$[AgSO_4]^-$	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Total	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

ERM (embryo rearing medium).

Table S2. Percentages of chemical species in $AgNO_3$ solutions, as calculated by using Visual MINTEQ version 3.0.

	1× ERM	5× ERM	10× ERM	15× ERM	20× ERM	30× ERM
Ag^+	0.9%	0.1%	0.0%	0.0%	0.0%	0.0%
$[AgCl]^0$	33.0%	8.2%	3.7%	2.2%	1.5%	0.9%
$[AgCl_2]^-$	64.5%	79.6%	72.5%	65.2%	59.2%	50.9%
$[AgCl_3]^{2-}$	1.6%	12.2%	23.8%	32.6%	39.2%	48.3%
AgOH	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
$[Ag(OH)_2]^-$	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
$[AgSO_4]^-$	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
$AgNO_3$	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Total	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

ERM (embryo rearing medium).

Table S3. Percentages of chemical species in silver solutions, as calculated by using stepwise formation constants for Cl^- and Ag^+ (%).

	1× ERM	5× ERM	10× ERM	15× ERM	20× ERM	30× ERM
Ag^+	2.1%	0.1%	0.0%	0.0%	0.0%	0.0%
$[AgCl]^0$	39.3%	9.1%	3.2%	1.4%	0.7%	0.3%
$[AgCl_2]^-$	55.8%	65.0%	45.0%	30.1%	20.9%	11.3%
$[AgCl_3]^{2-}$	2.4%	14.0%	19.4%	19.5%	18.0%	14.7%
$[AgCl_4]^{3-}$	0.4%	11.7%	32.4%	48.9%	60.3%	73.7%
Total	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

ERM (embryo rearing medium).

Table S4. Diameters of medaka eggs incubated in embryo rearing medium (ERM) at different concentrations.

ERM concentration	Egg diameter (mm)	Mean (mm)	SD (mm)
1×	1.30 1.25 1.30	1.29	0.03
5×	1.30 1.25 1.28	1.28	0.02
10×	1.30 1.30 1.33	1.31	0.01
15×	1.25 1.23 1.30	1.26	0.04
20×	1.30 1.28 1.30	1.30	0.01
30×	1.30 1.25 1.30	1.29	0.03

Formula S1

$$\rho = R \cdot (4\pi r^2/L)$$

ρ: electrical resistance of egg chorion

R: resistance (electrical current / voltage)

π: ratio of circumference of a circle to its diameter

r: radius of medaka egg

L: length of conductor (diameter of medaka egg)

References

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