Benefits of controlled copper ion delivery from copper nanoparticles (CuNP) to elastin matrix synthesis & maturation

Anand Ramamurthi, PhD, Chandrasekhar Kothapalli, MS
Clemson University-Medical University of South Carolina Bioengineering Program, Charleston, SC

Statement of Purpose: Elastin critically maintains native vessel structure and regulates cell-signaling pathways involved in morphogenesis, injury response, and inflammation. Unfortunately, elastin regeneration in situ or within tissue engineered constructs is challenged by very poor elastin matrix synthesis by adult vascular cells, and inadequacy of known cellular cues to up-regulate the same. We have shown that hyaluronan (HA) tetramers (HA4 mer; MW: 756 Da; 0.2 μg/ml) upregulate elastin precursor (1.82 ± 0.26 times vs. non-HA controls) and crosslinked matrix synthesis (7.5 ± 1.5-fold) by healthy, adult rat vascular smooth muscle cells (SMCs), enhance elastin matrix yield (8.7 ± 0.02 % vs. 5.3 ± 0.01% of total elastin for controls), maturation, stability against enzymatic degradation, and fiber formation. While these results benefit tissue engineering elastin rich constructs, elastin matrix yield is still very poor, while elastin fiber formation can be further improved. We thus need to identify and optimize '**elastin assembly/ maturation cues'** to improve these parameters. Possible cues are copper ions (Cu^{2+}) that are essential for function of lysyl oxidase (LOX) an elastin crosslinking enzyme. Here, we seek to compare benefits of two modes of Cu^{2+} delivery: constant doses delivered from soluble $CuSO_4$, and increasing but equivalent overall release from CuNP.

Methods: We investigated effects of Cu^{2+} delivery in presence and absence of HA 4mers (0.2 μg/ml). Delivered $CuSO_4$ doses (0.01 and 0.1 M) were selected based on that described in unrelated literature in context of enhancing LOX production. Sodium sulfate (Na_2SO_4; 0.1 M/0.01 M) was added as a negative control to exclude SO_4^{2-} ions as a source of any observed effects. The dose of CuNP **(**80-100 nm; MW = 63.55) were selected such as to generate an equivalent of the higher $CuSO_4$ dose (0.1 M; see below), which we found to proffer some benefit to elastin matrix deposition. To determine this dose, we first ascertained kinetics of Cu^{2+} release from CuNP (1, 10, 100 ng/ml; n = 3/conc.) over 30 days, in serum-rich PBS. Cu^{2+} concentrations were measured using atomic absorption spectroscopy and cumulative Cu^{2+} release calculated. The effects of CuNP dose (ng/ml) and time on the Cu^{2+} released was fit using a hierarchial regression analysis, wherein a new predictor is iteratively added to or dropped based on the statistical significance ($p<0.05$) of a quadratic regression model which accommodates linear, curvature & inter-dependence of time (x) and CuNP dose (y). Based on this analysis, we predicted that a CuNP dose of 400 ng/ 5mL released the equivalent of a steady 0.1 M-dose of $CuSO_4$, over 21 days. Healthy rat aortic SMCs (passage 3-6) were cultured with the respective additives for 21 days. At this time, the cell layers were assayed for DNA (flurometric assay), tropoelastin, and alkali-soluble and –insoluble matrix elastin (Fastin assay) and collagen (Hydroxyproline assay). Desmosine crosslinks (ng/ng of matrix elastin) were quantified using ELISA. Matrix organization was compared via SEM. LOX was quantified by western blot and its activity with an Amplex Red assay. **Biochemical outcomes were normalized to DNA content of the respective cultures**.

Results: Na_2SO_4 had no effect on cell behavior or matrix synthesis. As seen in **Table 1 (bottom right)**, a $CuSO_4$ dose of 0.1M (but not 0.01M; **see bottom left**) enhanced LOX production and activity, more so when HA 4 mers were also present. Perhaps via LOX mediation, this dose of Cu^{2+} also appeared to enhance tropoelastin and matrix elastin synthesis on a per cell basis, relative to non-additive controls. However, this dose (0.1 M) of $CuSO_4$ was also mildly cytotoxic and caused chronic but partial cell death to result in a net decrease in proliferation wrt controls. Thus, there is uncertainty in determining the actual cell number that contributed to matrix production, due to which the matrix production data **(see red box in Table)** may be calculated much higher than actually so. However, the matrix yield, which is independent of cell number, was unchanged wrt controls (28.6 ± 3.9%), suggesting no benefits to tropoelastin recruitment & crosslinking. Encouragingly, when delivered with HA 4mers CuNP did not impact cell proliferation, was not cytotoxic, had no impact on total elastin output on a per cell basis, but enhanced amounts (per ng of DNA) and yields of matrix elastin (58.8 ± 6.1% vs. 28.6 ± 3.9% for controls), possibly by enhancing LOX synthesis, activity (**Table 1; top unshaded column**). With or without HA 4mers, both Cu^{2+} delivery methods prompted robust elastic fiber deposition, more than in HA 4mer-only cultures. SEM of alkali-digested cell layers showed elastin in $CuSO_4$-added cultures to be organized into sheets, with aggregates of meshing fibers distinctly visible in regions. Elastin in cell layers cultured with CuNP was consistently more fibrous, porous than $CuSO_4$–added cultures, with individual fibers clearly seen**.**

CuNP dose	400 ng of CuNP ⇔ 0.1 M of Cu^{2+} release	
	No HA	HA 4-mer
DNA	1.32 ± 0.16	0.92 ± 0.20
Tropoelastin	0.40 ± 0.01*	0.64 ± 0.14*
Matrix Elastin	1.50 ± 0.08*	2.28 ± 0.23*
Total Elastin	0.75 ± 0.03*	1.11 ± 0.07*
Matrix/ Total elastin	2.13 ± 0.12*	2.05 ± 0.21*
LOX activity	1.01 ± 0.02	1.13 ± 0.02*
LOX synthesis	1.09 ± 0.14	1.20 ± 0.10*

$CuSO_4$ dose	0.01 M		0.1 M	
	No HA	HA 4-mer	No HA	HA 4-mer
DNA	1.94 ± 0.3*	1.40 ± 0.2*	0.24 ±0.1*	0.20 ± 0.1*
Tropoelastin	0.51±0.01*	0.73 ± 0.1*	4.14 ±0.1	5.44 ± 0.7
Matrix Elastin	0.60 ± 0.1*	1.23 ± 0.3	4.20 ± 0.7	5.80 ± 0.8
Total Elastin	0.5 ± 0.02*	0.74 ± 0.0*	4.13 ± 0.1	5.24 ± 0.3
Matrix/ Total elastin	0.98 ± 0.1	1.04 ± 0.04	1.00 ± 0.0	0.86 ± 0.1
LOX activity	1.40 ± 0.2*	1.12 ± 0.14	1.09 ± 0.2	1.19 ± 0.03*
LOX synthesis	0.76 ± 0.1*	0.92 ± 0.10	1.30 ± 0.2*	1.35 ± 0.1*

Table 1. Matrix production data are normalized to DNA content of respective cultures at 21 d. All data are **normalized to additive-free controls**. n = 3/case; * p <0.05 for significance.

Conclusions: For identical total Cu^{2+} release, relative to $CuSO_4$, CuNP more efficiently delivers Cu^{2+} for localized activity at the cell layer to enhance elastin matrix yield, LOX synthesis and activity, and elastin fiber formation. Cu^{2+} enhancement of LOX and elastin are also more pronounced in presence of HA 4mers. Though optimization of CuNP is necessary, they are a useful tool to enhance elastin matrix quality and yield in consort with other elastogenic cues. [**Support:** NIH; EB006078-01A1]