96-well nephelometric assay to detect calcification propensity of serum samples

A. Pasch[1] and W. Jahnen-Dechent[2]
[1]University of Bern, Bern, Switzerland; [2]RWTH Universitätsklinikum, Aachen, Germany

- **Cardiovascular diseases are related to vascular calcification**
- **Fetuin-A is a strong calcification inhibitor**
- **Assay can be applied to serum samples from patients and healthy individuals**

Introduction

Calcium ions (Ca^{2+}) and phosphate are used by the human body to build the structural material of bone and teeth. The resulting crystalline calcium phosphate is the product of the calcification process. Concentrations of Ca^{2+} and phosphate are close to supersaturation in most tissues and body fluids, and as a result precipitation could happen anywhere in the body. In healthy people this is usually not the case and so it is comprehensible that the calcification process must be very strictly regulated. If this regulation is not working properly, calcification can also take place in soft tissues or blood vessels, leading e.g. to cardiovascular diseases, the most predominant cause of death all over the world.

The serum protein fetuin-A is a potent calcification inhibitor. Together with additional blood components, fetuin-A will prevent that Ca^{2+} and phosphate precipitate despite supersaturation. Small colloidal protein-mineral complexes are generated instead. These primary calciprotein particles (CPP) will spontaneously convert to crystalline secondary CPP while changing in shape and particle diameter. The transition happens in a timed and coordinated manner and is thought to reflect the intrinsic inhibitory calcification propensity of a given fluid. In this application note we describe a test that measures the transition from primary to secondary CPP in serum samples. This label-free 96-well plate-based assay measures the conversion of primary to secondary CPP by detecting the changes in laser light scattering associated with it. We used a NEPHELOstar® *Plus* microplate reader from BMG LABTECH.

Assay Principle

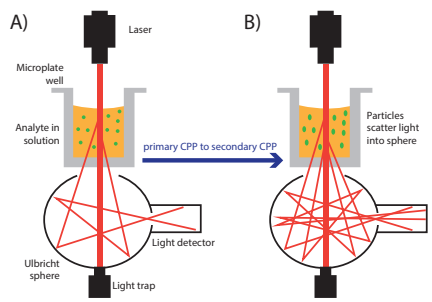

Fig. 1: Nephelometric assay principle.

A laser-generated light beam is passed through a sample. The particles in solution will scatter light depending on particle size and/or shape. Primary CPP and secondary CPP have different shapes and particle diameters enabling the NEPHELOstar *Plus* to measure both states. The forward scattered light is detected at an angle up to 80 degrees.

Materials & Methods

- NEPHELOstar *Plus*, BMG LABTECH
- Liquidator96 bench-top pipetting system, Mettler Toledo
- clear 96-well plates from Brand
- 96-well adhesive plastic cover sheets from Carl Roth
- all chemicals from AppliChem, Sigma or Roth

Serum Samples

Serum samples from healthy volunteers as well as serum samples from hemodialysis patients were assayed. It has been shown that patients that undergo hemodialysis have an increased risk for accelerated vascular and soft tissue calcifications. Mouse sera was prepared from DBA/2 fetuin-A-deficient (-/-), heterozygous (+/-) and wild-type (+/+) mice.

Microplate Preparation

All liquids were pipetted using the Liquidator96 benchtop pipetting system in the following order (volumes for one well)

- 20 µl NaCl solution (140 mM NaCl)
- 80 µl serum
- Mix 1 minute using a vibrating shaker
- 50 µl phosphate solution (19.44 mM Na_2HPO_4+4.56 mM NaH_2PO_4+100 mM Hepes+140 mM NaCl pH-adjusted with 10 M NaOH to 7.40 at 37°C)
- Mix 1 minute using a vibrating shaker
- 50 µl calcium solution (40 mM $CaCl_2$+100 mM Hepes+ 140 mM NaCl pH-adjusted with 10 M NaOH to 7.40 at 37°C)
- Mix 1 minute using a vibrating shaker
- Cover 96-well plate using adhesive sheets

NEPHELOstar instrument settings

The plate was put into the NEPHELOstar and read using the following parameters:

Plate mode kinetic	
No of cycles:	200
Measurement time:	1.5 seconds
Cycle time:	180 seconds per cycle
Positioning delay:	0.1 seconds
Laser beam focus:	1.5 mm
Laser intensity:	50 %

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Total assay run time was 10 hours, but some measurements were also longer. To minimize temperature fluctuations during measurements, the internal NEPHELOstar temperature control was turned off and all measurements took place in a thermostated room at 34.5°C, leading to an internal temperature of 36.5-37°C in the NEPHELOstar.

Results & Discussion

Using three-dimensional dynamic light scattering (3D-DLS), we determined the hydrodynamic radius Rh for primary CPP and secondary CPP. CPP diameters were measured both in test solutions containing fetuin-A, phosphate and Ca^{2+} as well as in serum samples. The resulting diameters were quite similar (primary CPPs were between 60 and 75 nm whereas secondary CPP were between 120 and 150 nm) but showed significantly different transition times (delayed in serum samples). We concluded that the delay of the conversion step can be related to the stability of primary CPP and that measuring this step will help to determine the inhibitory potency inherent in serum samples. Optimization of assay and all further measurements were done with the NEPHELOstar. Fig. 2 shows a measurement over time while primary CPP convert to secondary CPP.

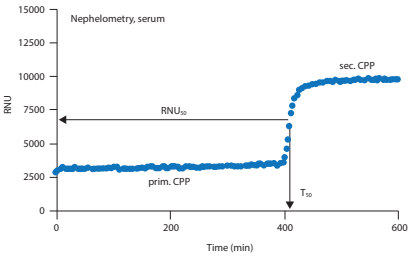

Fig. 2: Primary to secondary CPP transformation step over time in the presence of medium.

After measurement, data were exported to Excel and GraphPad prism for data evaluation. The one-half maximal transition time (T_{50}) and the one-half maximal nephelometric units (RNU_{50}) were calculated (Fig. 2). For assay validation, mouse and human samples were measured. Figure 3 shows that the assay could discriminate between mouse sera derived from wild type and fetuin-A-deficient sera. The nephelometric results agreed well with the calcification load observed of these mice.

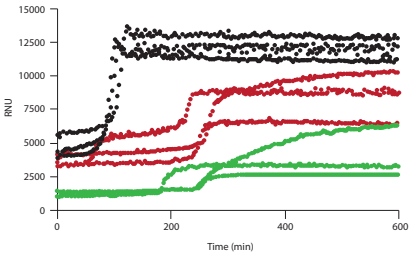

Fig. 3: Nephelometry assay using sera from adult 10- to 16-month-old noncalcifying wild-type DBA/2 mice (green), noncalcifying heterozygous fetuin-A+/2 knockout mice having half-normal serum fetuin-A (red), and heavily calcifying fetuin-A–deficient homozygous fetuin-A2/2 knockout mice (black).

Human serum samples were obtained from healthy donors and hemodialysis patients. Fig. 4 shows the results for both groups.

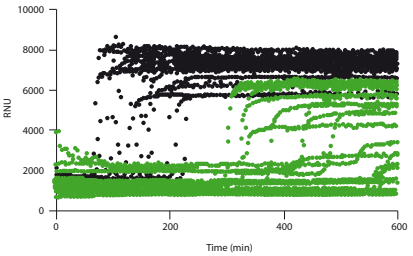

Fig. 4: Nephelometry assay with sera from 20 hemodialysis patients (black) and 20 healthy volunteers (green).

Again the test was able to distinguish between the two donor groups indicating the utility of the test for measurement of human serum samples. Our results demonstrate that the nephelometer test is useful to measure calcification propensity in body fluids with reasonable sample throughput.

Conclusion

Our test is a novel 96-well nephelometer-based assay which measures the overall calcification risk in serum. As calcification is an often observed process in a lot of diseases this assay may be used in clinical and basic research. The routine clinical use of the test requires evaluation in a prospective study.