Changes to the structure of blood clots formed in the presence of fine particulate matter

Sofian Metassan¹,², Robert A S Ariens³, D Julian Scott³ and Michael N Routledge¹

¹Molecular Epidemiology Unit and
²Cardiovascular and Diabetes Research Division, Leeds Institute for Health, Genetics and Therapeutics, The LIGHT Laboratories, University of Leeds, Leeds LS2 9JT

Email: umphsp@leeds.ac.uk

Abstract. Both long-term and short-term exposure (one to two hours) to particulate matter are associated with morbidity and mortality caused by cardiovascular diseases. The underlying mechanisms leading to cardiovascular events are unclear, however, changes to blood coagulability upon exposure to ultrafine particulate matter (UFPM, the smallest of which can enter the circulation) is a plausible mechanism. Objectives: This study aims to investigate the direct effects of particulate matter on fibrin polymerization, lateral aggregation and the formation of fibrin network structure. Methods: Standard Urban Particulate Matter (PM) was suspended in Tris buffer centrifuged and filtered with <200nm filter to obtain ultrafine PM or their water-soluble components. Purified normal fibrinogen was made to clot by adding thrombin and calcium chloride in the presence of varying concentrations of PM. Permeation properties (Darcy constant [Ks]) and turbidity of clots were measured to investigate the effects on flow-rate, pore size, and fibrin polymerization. In addition, confocal microscopy was performed to study detailed clot structure. Results: Total PM increased the Ks of clots in a dose dependant manner (Ks = 4.4, 6.9 and 13.2 x 10⁻⁹ cm² for 0, 50 and 100 µg/ml total PM concentrations, respectively). Filtered PM also produced a significant increase in Ks at PM concentration of 17 µg/ml. Final turbidity measurements at 20min were obtained for varying concentrations of PM. Maximum optical density (OD) for 1 mg/ml fibrinogen at 0, 50, 100 and 200 µg/ml total PM concentrations were 0.39, 0.42, 0.45 and 0.46, respectively. The maximum OD for 0, 17, 34 and 68 µg/ml filtered PM concentrations were 0.39, 0.42 0.47 and 0.51, respectively, suggesting an increase in fibre diameter with increasing particulate concentration. The lag phase was significantly shorter and the rate of polymerisation was significantly faster in the presence of 68 µg/ml filtered PM. Confocal microscopy results showed decrease in fibre density without a significant increase in fibre diameter in the presence of total PM and filtered PM. Conclusion. The results indicate that total PM and filtered PM are capable of causing alterations to the fibrin polymerization and network structure as shown by the changes in permeation properties, the turbidity experiments as well as by confocal microscopy.

1. Background and objectives
Epidemiological studies have shown that exposure to air pollution is associated with increased hospitalisation and mortality due to cardiovascular disease [1-3]. Peters et al. showed that recent
exposure to air pollution was associated with myocardial infarction with an odds ratio of 2.92 (95% Confidence Interval (95% CI), 2.22 to 3.83, p<0.001) [4]. Air pollution constitutes gaseous pollutants and particulate matter (PM) of varying sizes. There is increasing evidence that suggests that exposure to varying concentrations of PM is associated with myocardial infarction (MI), coronary heart disease (CHD), arrhythmia and cerebrovascular disease [5, 6]. The main source of traffic related PM is from diesel and petrol combustion engines, although road surface, tyre and brake wear all contribute to PM. PM has been classified according to size. PM_{10}, PM_{2.5} and PM_{0.1} are particles less than 10 μm, 2.5 μm and 0.1 μm in aerodynamic diameter, respectively. The particles consist mainly of elemental carbon (carbon black or soot), with small amounts of trace metals (such as lead, zinc, iron and aluminium), sulphate salts and nitrate salts. There may also be traces of varying compositions of polycyclic aromatic hydrocarbons. The size of the particles determines their ability to penetrate deep into the lungs, finer particles reaching the bronchioles and the alveoli. In order to enter the blood circulation, particles have to pass through the air-blood barrier, which is only 0.5 μm thick, and is where gaseous exchange takes place. Ultrafine particles (UFP; <0.2 μm) have been found capable of crossing the cellular membrane of the alveoli without the need for endocytosis or any form of receptor-mediated, actin-based process. In the lungs, the particles have been found in the tissue compartments, cells and within capillaries [7]. The presence of UFP in blood has been shown in vivo [8, 9].

One of the most important plasma proteins during the coagulation process is fibrinogen. The final step in the formation of clot is the conversion of fibrinogen to fibrin which polymerizes into a three-dimensional network. The polymerization of fibrinogen is initiated by the action of thrombin to cause the conversion of fibrinogen to fibrin. The fibrin clot is broken down by the process of fibrinolysis through the action of plasmin. During clot formation, plasminogen is bound to fibrin. Tissue plasminogen activator (t-PA) converts plasminogen to plasmin, initiating fibrinolysis. The rate of fibrinolysis may depend on fibrin fibre diameter and fibrin clot structure. Fibrin clot with a dense structure made of thin fibres dissolved more slowly than coarse fibrin clot structure made of thicker fibres. The formation of abnormal structure of fibrin clot has been linked to the thrombosis[10-12]. People with myocardial infarction are more likely to have tighter and more rigid fibrin clot network with lower permeability compared to normal subjects [13]. According to confocal microscopy data, plasma clots from coronary artery disease (CAD) patients were made of fibrin fibres that were shorter, thinner and more numerous than clots from normal control subjects. Furthermore, the fibres from the CAD patients were more resistant to lysis with a slower rate of lysis than that of control subjects [14]. In this study we have studied the characteristics of blood clots formed in vitro in the presence of PM and UFP.

2. Study description

2.1. Particulate matter

The PM used was a Standard Reference Material (SRM) 1648 purchased from National Institute of Standards and Technology (NIST). The SRM was collected from St Louis, MO, United States, accumulated over a period of over 12 months. The SRM mainly consists of carbon particles with various elements such as aluminium, iron, potassium, lead, sodium and zinc. Two portions of the SRM were suspended in Tris buffer (0.05M Tris-HCl, 0.1M NaCl, pH7.5) at concentration of 5mg/ml. One portion was kept aside as total particulate matter (TPM). Another portion was centrifuged at 13,400 rpm for 30 minutes and the supernatant filtered with a serological 0.22 μm filter with a hydrophilic polyethersulfone membrane (Millex GP, Millipore). This portion was called filtered particulate matter (FPM).

2.2. Fibrin permeation

Purified fibrinogen (100 μl) was made to clot by adding 10 μl of activation mixture (thrombin and calcium chloride). The final concentrations were 1 mg/ml, 1 U/ml and 10 mM for fibrinogen,
thrombin and calcium chloride, respectively. Immediately after mixing, 100 μl of the clotting mixture was transferred to a plastic pipette tip that had been prepared by carefully cutting 4.5cm off the tip and making the interior surface rough. The clotting mixture was kept in a moist chamber for 2 hours at room temperature. The clot was then washed by allowing 6 drops of the Tris buffer to pass through. The time taken for 6 more drops were recorded and the weight of the drops measured. The Darcy’s constant (Ks), which is a measure of the pore size of fibrin network through which liquid may flow [11] was calculated.

2.3. Turbidity measurements
The stages of fibrin polymerisation were followed by turbidity measurements. The Lag phase measures the protofibril formation. Rate of polymerisation (Vmax) measures the lateral aggregation and fibre formation. The maximum optical density (OD) gives the information on fibre diameter.

2.4. Laser scanning confocal microscopy
Confocal microscopy was performed on clots formed in a confocal microscope slide chamber. A Leica TCS-SP2 confocal system was used with an inverted microscope (Leica DMIRE2), equipped with a 63X water immersion objective lens using the reflection mode. The scan format was set at 512x512 pixels. The pinhole set to one Airy unit to obtain maximum resolution in the z-plane. The fibre diameters were measured by using the software (Quantification Tools - Profile). The scans were taken using 8x zoom-in mode (30 x 30 μm). A line was drawn across the fibre avoiding any junction and the length of the line was recorded. Five fibre diameters were measured for each scan. The fibre density for each scan was calculated as follows: the scans were taken at 4x zoom-in mode (60 x 60 μm). A line was drawn across the scanned image, from right to left and the number of fibres crossing the line were counted.

2.5. Statistical methods
Statistical significance was determined by ANOVA followed by Bonferroni’s test. The level of significance was taken as p<0.05. Results were express as mean ± 95% confidence interval (95% CI). Some results were also expressed as mean ± standard error mean (SEM).

3. Results

3.1. Fibrin permeation
As it is thought that UFP can enter the blood, the TPM was centrifuged and filtered to remove particles larger than 220 nm (FPM). Both FPM and TPM were lyophilised and the weight measured. Distilled water was used as a blank control. The percentage of the FPM was calculated to be 34% of the TPM. This value was used to calculate the actual concentrations of filtered PM. Ks of TPM at concentration of 100 μg/ml was significantly different than control (p<0.05). In a similar experiment as for TPM, FPM caused a significant effect on the fibrin clot permeability. As FPM was increased from 0 μg/ml to 17 μg/ml, there was a significant increase in the mean Ks (3.74 and 8.29 x 10^{-9} cm^{2} for 0 and 17 μg/ml FPM, respectively, p<0.05) (Figure 1). The graph was plotted as Darcy constant (Ks) against total PM concentration. Every point represents the mean and 95% confidence interval. (A) 0, 50 and 100 μg/ml total PM. (B) 0, 17 and 34 μg/ml filtered PM.

3.2. Turbidity measurements
When fibrinogen was incubated with increasing concentrations of FPM, there was also a corresponding increase in the maximum OD (mean= 0.39, 0.42, 0.47 and 0.51 for control, 17, 34 and 68 μg/ml of FPM, respectively; Fig 2). The OD at concentrations of 34 and 68 μg/ml were significantly different from control (p<0.05). Similar results were seen for TPM. The lag phase, the
rate of protofibril formation, was measured and found to be significantly reduced in the presence of 68 mg/ml FPM (39.4 vs 12 sec for control vs filtered PM). Vmax, the rate of polymerisation, was also measured and shown to be increased in the presence of 68 mg/ml FPM (1.4 vs $2.3 \times 10^{-3}$ sec$^{-1}$) for control vs FPM) (Table 1). Therefore, the presence of FPM caused faster formation of half-staggered double-stranded protofibril as well as the formation of lateral aggregation of the protofibrils leading to the formation of fibrin fibres.

![Figure 1](image1.png)

**Figure 1** Permeation of 1 mg/ml fibrinogen clot in the presence of PM.

| Lag phase (s) | Vmax ($10^{-3}$ s$^{-1}$) |
|--------------|--------------------------|
| Control      | 39.4 ± 6.3 (n=4)         |
| 200 μg/ml TPM| 21.0 ± 9.0 (n=4)         |
| 68 μg/ml FPM | 12.0 ± 0.0 (n=4)         |

The values were mean ± SEM. The Lag phase and Vmax of 68 μg/ml Filtered PM are statistically significant from control (p<0.05).

The changes to the fibrin network structure were investigated in detail by digital measurements of fibre density and fibre diameter using LSCM performed at increasing concentrations of TPM and FPM (fig 3). The mean of fibre density was calculated from the LSCM scans and plotted against total PM concentration. Total PM caused decrease in the fibre density (mean = 9.1, 8.2, 8.3, 7.2 fibres/60μm for control, 50, 100 and 200 μg/ml of TPM). Fibre density at 200 μg/ml TPM concentration was significantly decreased from control (p< 0.05). FPM also caused a significant decrease in fibre density.

Fibre densities for 17, 34 and 340 μg/ml of FPM were statistically significant (p<0.05). In contrast to fibre density, there was no significant change in the fibre diameter with increasing concentration of either TM or FM. Also, the presence of high concentration of FPM (340 μg/ml) did not cause any significant change to the fibre diameter.
Figure 2. Effects of filtered PM fibrin polymerization, measured by turbidity. Clots were formed with 1 mg/ml fibrinogen, 0.25 U/ml thrombin and 10 mM calcium chloride. Each point represents mean and 95% CI. (A) 0, 50, 100 and 200 μg/ml total PM. (B) 0, 17, 34 and 68 μg/ml filtered PM. The OD for total PM concentrations of 100 and 200 μg/ml as well as filtered PM concentrations of 34 and 68 μg/ml were statistically significant from control (p<0.05).

Figure 3. Fibre density in the presence of total PM and filtered PM, measured from confocal microscopy scans. Clots were formed with 1.0 mg/ml fibrinogen, 1 U/ml thrombin and 10 mM calcium chloride. (A) Presence of total PM. (B) Presence of filtered PM. Each point was plotted as mean and 95% CI.
4. Conclusions

Fibrinogen is central to the formation of thrombus. Alteration to the fibrinogen structure and function, changes to fibrin polymerisation, formation of heterogeneous clot structure due to the formation of clustered fibrin fibres might all add up to the formation of an unstable thrombus. Our findings indicate that filtered PM which contained soluble components (such as metal ions) as well as UFP particles of less than 0.22 μm in diameter were capable of causing alterations to fibrin network properties, with changes in fibre density, network structure and clot permeability. However, independent effects of UFP have not been studied. Some of these changes may contribute to the observed health effects of airborne PM on cardiovascular function.

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