CHEMICAL ENGINEERING TRANSACTIONS
VOL. 32, 2013
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at: www.aidic.it/cet
Chief Editors: Sauro Pierucci, Jiří J. Klemeš

**ISBN** 978-88-95608-23-5; **ISSN** 1974-9791
DOI: 10.3303/CET1332377

# Spraying of Cell Colloids in Medical Atomizers

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Aerosols are conveniently used for delivery of medicines to the human body. The most common application is inhalation of anti-asthmatic or anti-inflammatory drugs, nevertheless the spectrum of therapeutic agents that may be administered via the respiratory system is continuously expanding. Aerosols are also used topically (on skin) as a method of speeding up the healing. In this context, bio-colloids (e.g., cell suspensions) are considered as the material for which the proper atomization method must be established to assure that local hydrodynamic stresses related to droplet formation will not destroy the living cells. In this work we test the influence of selected spraying techniques on the integrity and survival of different types of cells after the atomization process using medical atomizers. Medical nebulizers and spray devices were characterized in respect of emitted droplet size distribution and used to aerosolize model bio-colloids (suspensions of yeast, bacteria and mouse fibroblasts). Cell viability after spraying was determined via direct microscopic observations and specific microbiological assays. Commonly used medical inhalers produce droplets which are too small (Dv50 ≈ 4-5.5 μm) to contain whole living cells. As generation of such fine droplets is related to high local hydrodynamic stresses, cell viability in the suspension is strongly reduced even for small and mechanically resistant cells. Nasal atomizer (spray pump) and mechnical Microsprayer produce larger droplets (Dv50 ≈ 50-80 $\mu$m) which are capable of carrying intact fibroblast cells. Due to a lower shear during atomization process in these devices, the cells remain viable and active. Based on the results of experimental study, only selected techniques can be proposed for bio-colloid atomization in medical applications.

## 1. Introduction

Atomization of colloidal suspensions containing living cells has potential applications in regenerative therapies. For instance, stem cells administered as an aerosol have been demonstrated to be successfully used in healing skin burns and scars (Gravante et al. 2007, Gerlach et al, 2011). Great expectations are related to the regenerative medicine of the lungs - the vital organ which can be easily damaged due to inhalation of hot or aggressive gases and vapours (Angelini et al, 2013). The natural way of delivery of therapeutic agents to the lungs is their conversion to aerosol which may be naturally inhaled and deposited inside the respiratory system. Commonly, anti-asthmatic and anti-inflammatory drugs are targeted to the lung tissue after aerosolization in different types of medical inhalers: metered dose inhalers (MDIs), dry powder inhalers (DPIs) or liquid atomizers, also known as nebulizers (Newman, 2009; Sosnowski, 2012). In this work we consider the use of aerosol as a carrying medium for transfer materials containing cells to the respiratory tract in order to regenerate damaged lung tissue. We focus on testing the applicability of selected medical atomizers for spraying colloids containing cells. This basic research should help in the rational selection of atomizing devices for cell delivery in the regenerative medicine of the lungs (decullarization followed by cell seeding - Petersen et al., 2011) but also of other organs (e.g., skin).

Please cite this article as: Sosnowski T., Kurowska A., Butruk B., Jablczynska K., 2013, Spraying of cell colloids in medical atomizers, Chemical Engineering Transactions, 32, 2257-2262 DOI: 10.3303/CET1332377

## 2. Materials and methods

### 2.1 Biological material

Three types of model cells were used in these studies:
a) yeast cells: *Saccharomyces cerevisiae* (Lessafre, Poland),
b) bacterial cells: *Lactobacillus casei* (Lek-AM, Poland),
c) animal (mammalian) cells: mouse fibroblasts, line L929 (European Collection of Cell Cultures, UK).

These cells are characterized by different size - yeast: 5-10 μm, bacteria: 2-4 μm, fibroblasts: 30-130 μm. The appropriate aqueous/buffer suspension of each type of cells was prepared just before atomization studies. Yeast and bacteria were studied in non-sterile conditions, while all investigations with fibroblasts required sterile conditions and media.

### 2.2 Atomizing devices and procedures for cell survival studies

Selected types of medical atomizers operating on different spraying principles have been used:
a) PN - pneumatic nebulizer RF6 (FlaemNuova, Italy) connected to MP1 medical compressor (Medbryt, Poland),
b) US - ultrasonic nebulizer Thomex (Medbryt, Poland),
c) VM - vibrating-mesh nebulizer AeroNeb (Aerogen Inc., Ireland),
d) MSA - Microsprayer® Aerosolizer model IA-1B (Penn-Century Inc., Wyndmor, PA, USA),
e) NA - nasal atomizer (Coster, Italy).

Devices PN, US and VM were electrically supplied and controlled while atomizers MSA and NA were operated manually. Cell survival during atomization in US device was evaluated by analyses done only for the aerosol which was recycled inside the nebulizer since collection of the emitted droplets in amounts required for cell counting was not realizable. In case of PN and VM atomizers it was possible to analyze cell viability both in the atomized liquid circulating inside the nebulizing vessel and in the liquid collected after capturing of emitted aerosol. For MSA and NA devices the cell survival was analyzed solely in the liquid emitted as aerosol.

### 2.3 Droplet size distribution determination

Size distribution of droplets emitted from the atomizing devices was measured with diffraction aerosol spectrometer Spraytec (size range of 100 nm - 2500 μm - Malvern Instruments, UK). In contrast to many other optical methods which require substantial dilution of aerosol, the diffraction spectrometry allows for on-line analysis of the raw cloud emitted from any aerosol source.

### 2.4 Cell integrity and viability assays

Different assays were used depending on the cells type:
a) for yeast – vital staining (methylene blue 0.01%) and microscopic evaluation using Thoma counting chamber,
b) for bacteria – microscopic evaluation of cell integrity in Thoma chamber,
c) for fibroblasts – three assays: (i) vital staining with trypane blue (0.04 %) after trypsinization, (ii) “Life-Dead” fluorescence test, and (iii) MTT colorimetric assay after 48 hr incubation of cells at 37°C.

To evaluate and compare cell viability, the relative number of viable cells, *RVC*, was defined:

$$RVC = \frac{N}{N_0} \cdot 100\% \quad (1)$$

where: $N$ – number fraction of viable cells in the given sample, $N_0$ – number fraction of viable cells in the initial (control) sample, i.e. without atomization. For bacteria, where viability could not be evaluated, the relative amount of intact (integral) cells, *RIC,* was compared:

$$RIC = \frac{K}{K_0} \cdot 100\% \quad (2)$$

where: $K$ – number of intact cells visible in the field of view (counted in Thoma chamber), $K_0$ – number of integral cells visible in the in the same field in the control sample, i.e. without atomization.
All cell countings and viability assays were done in the required number of repetitions to obtain the confident average and standard deviation data.

## 3. Results

### 3.1 Droplet size distribution

Droplet size distribution in the aerosol emitted from different atomizers is depicted in Figure 1. Based on these data it is improbable that large cells (such as fibroblasts) can be sprayed without harm in the nebulizers (PN, US, VM) for which all droplets are smaller than 20 μm.

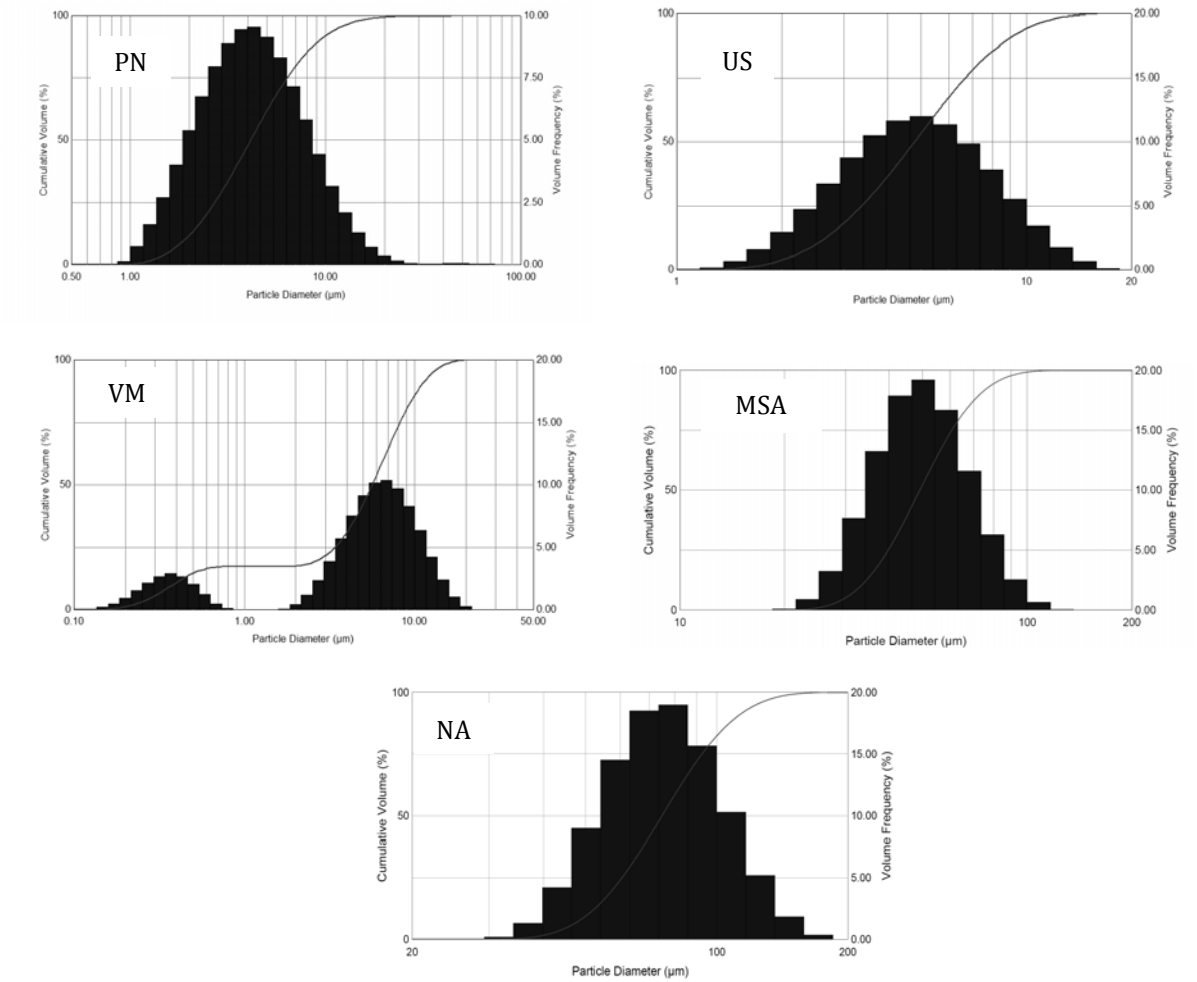


*Figure 1: Size distribution of droplets produced by all tested atomizing devices – designations as defined in chapter 2.2.*

Therefore, in the further cell survival studies we applied these three nebulization methods only for yeast and bacteria (average cell size below 10 μm) to test if hydrodynamic stresses associated with the spraying technique do not damage both types of cells. The obtained results seem to be positive as the approach does not damage the cells, thus suggesting some possibilities of application of such atomization devices to spray cell suspensions for regenerative purposes (e.g. delivery of stem cells). However, it must be kept in mind that bacteria and yeast have more resistant cell wall than animal cells (e.g. Touhami al, 2003).

Two other atomizing techniques (MSA, NA) allow to obtain significantly larger droplets (30-150 μm) so they seem more suitable for spraying of biocolloids containing larger and more sensitive animal cells because the hydrodynamic stresses of atomization should be weaker. The important practical problem in lung regeneration concept is limited to suitability of coarse droplets for cell targeting into the lungs. Typically, droplets/particles larger than 10 μm have a very low chance to penetrate beyond upper airways during normal inhalation (Sosnowski et al., 2006). Our recent studies indicate, however, that intratracheal aerosolization of large droplets may allow for their delivery to the bronchial tree.

### 3.2 Survival of yeast and bacterial cells during atomization in medical nebulizers

Cell survival during pneumatic (PN) atomization

Figure 2 illustrates the relative fraction of viable yeast cells, RVC, in the liquid which contains aerosol recycled in the pneumatic nebulizer (Figure 2a) and in the liquid which was emitted as aerosol droplets from this device (Figure 2b). Despite a rather large scatter of data, it is visible that yeasts are generally resistant to the stresses generated during atomization in PN device. After 6-8 minutes of spraying, 90% of viable cells loaded into the atomizer are still alive, and this rate of survival is only slightly lower (80%) for cells collected in the emitted aerosol. Survival of bacteria in the same nebulizer are shown in Figure 3, and it can be seen that the fraction of intact cells is noticeably lower – only 20% inside the vessel and almost none in the aerosol after 6 minutes of atomization. Due to high mortality of bacterial cells and low size of produced droplets, this atomizer was not used in the further tests with fibroblasts.

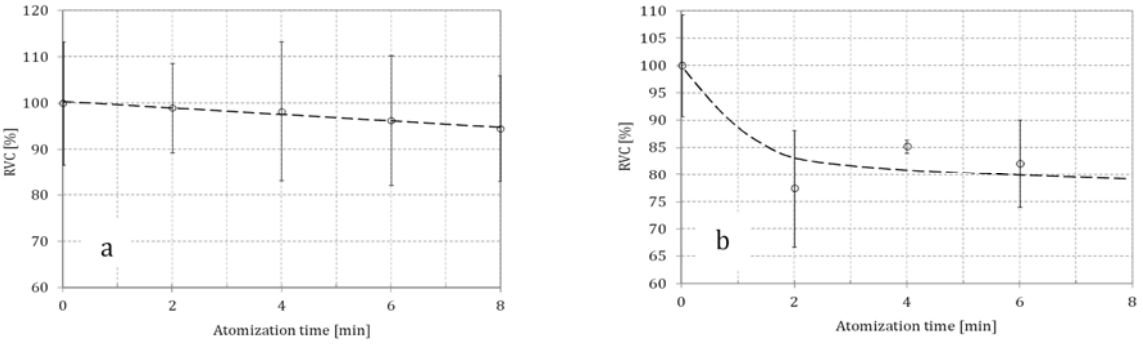


*Figure 2: Survival of yeast cells during pneumatic (PN) atomization: a – viable cells in the liquid inside the vessel, b – viable cells in the liquid emitted as droplets.*

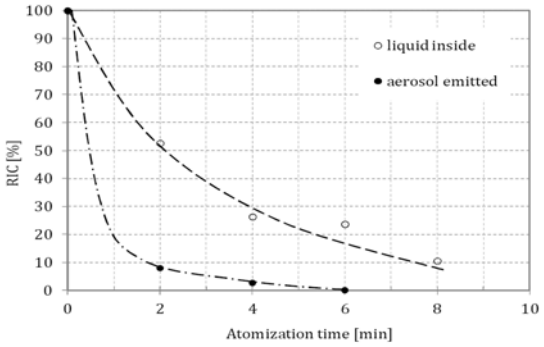


*Figure 3: Survival of bacterial cells during pneumatic (PN) atomization.*

Cell survival during ultrasonic (US) atomization

The data on cell viability during ultrasonic atomization are shown in Figure 4. Both yeasts and bacteria are noticeably destroyed by the stresses generated by ultrasounds. The transient increase of the viable yeast cells (Figure 4a) may be related to stress-induced reproduction (i.e. budding).Taking into account also the size distribution of droplets produced in this device (Figure 1), it is evident that US nebulizer cannot be used for carrying animal cell which are more sensitive and often larger.

Cell survival during atomization in the vibrating mesh (VM) nebulizer

The data on cell viability in VM atomizer are shown in Figures 5 (yeast) and 6 (bacteria). In both cases a significant reduction of the fraction of viable cells is observed as a function of spraying time. On the same rationale as US nebulizer, the VM device is inappropriate for atomization of animal cells.

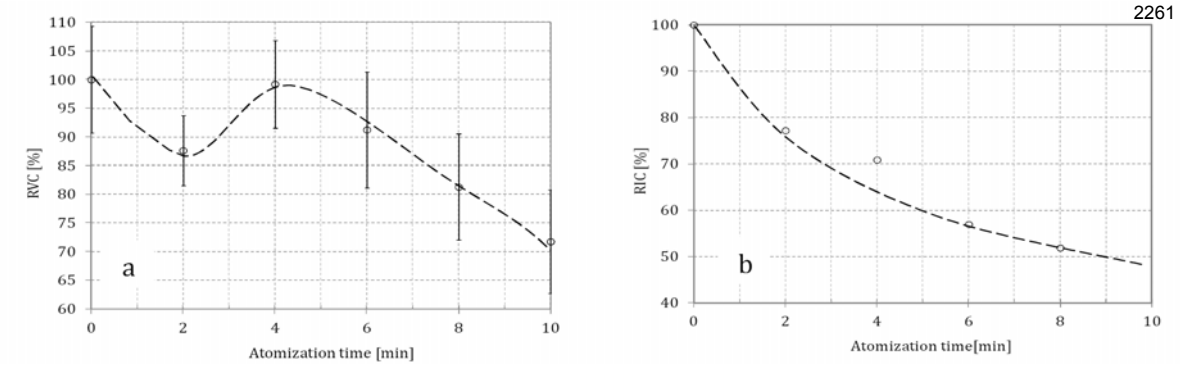


*Figure 4: Survival of cells during ultrasonic (US) atomization - cells collected from the aerosol recirculated inside the nebulizer: a - yeast, b - bacteria.*

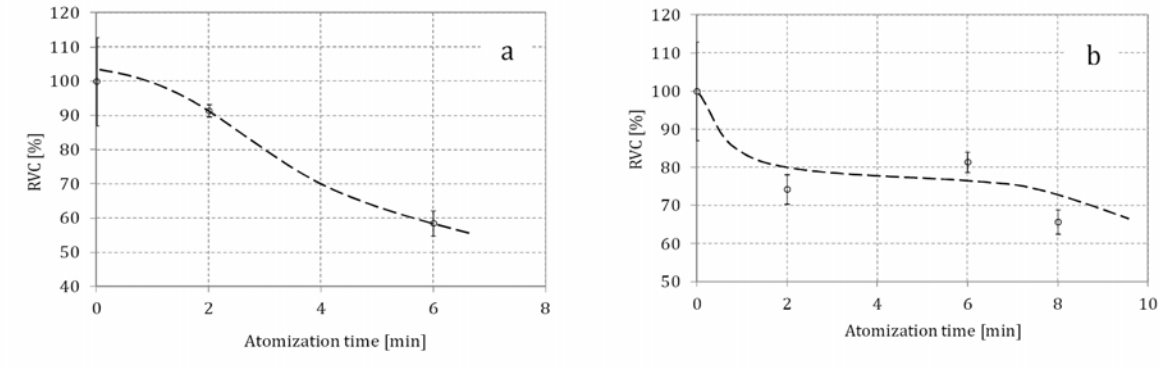


*Figure 5: Survival of yeast cells during VM atomization: a – in the liquid inside the vessel, b – in the liquid emitted as droplets.*

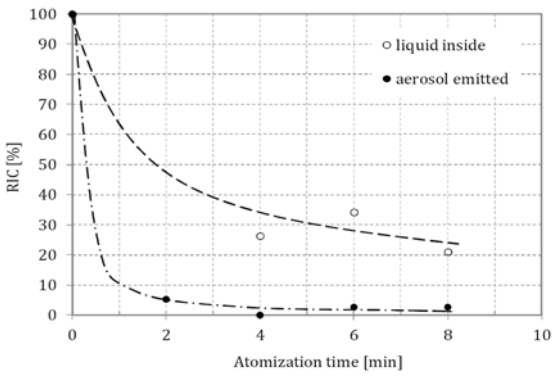


*Figure 6: Survival of bacterial cells during VM atomization.*

Survival of fibroblasts

Due to a large size of these cells, all common nebulizing techniques (pneumatic, ultrasound, VM) had to be excluded as they generate too small droplets to contain the entire fibroblasts. Therefore only two other atomization methods (MSA and NA) which allow to obtain droplets approaching 100 μm have been tested. As stated in section 2, survival rate for animal cells was determined using more complicated assays in sterile conditions. Figure 7 summarizes the results of 3 different tests. In all assays the survival rate of fibroblast cells was high (above 94%) indicating that both MSA and NA atomization techniques are suitable for spraying viable animal cells. The enzymatic MTT assay done after 48 h of incubation of fibroblasts sprayed from the nasal atomizer indicated a higher loss of cell activity (65% of the control), suggesting that such method of atomization is more destructive for these cells and may lead to their death over time. This effect was not observed for fibroblasts atomized with MA (Microsprayer) device.

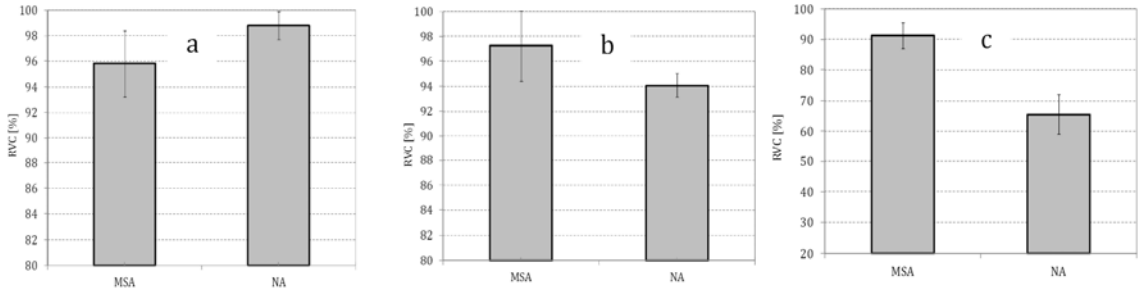


*Figure 7: Survival of fibroblasts atomized in MA and NA devices determined by different assays: a) "Life-Dead" fluorescence test, b) Trypan blue staining, c) MTT test (48 h after spraying).*

## 4. Discussion and conclusions

The experimental results indicate that nebulization techniques commonly used for atomization of drugs intended for inhalation are not suitable for spraying animal cells. The average droplet size (in the order of 5 μm) is too small to surround the whole cell so the biological colloid cannot be transferred to aerosol phase without inactivation. At the same time, the stresses required to generate such small droplets are very high, so even smaller and more resistant cells (e.g. bacteria) are damaged. Although some literature data indicate that cells can be very deformable without damage of their wall structure (Suchecka et al. 2005), the current results neglect the possibility that cells will survive the nebulization process. Two other atomization devices (Microsprayer and nasal atomizer) which generate lower hydrodynamic stresses to produce larger droplets were demonstrated to efficiently atomize the bio-colloid without significant damage of fibroblasts. Such large droplets are suitable for transferring cells to the surface of the body (skin), but they are inappropriate for lung targeting during normal breathing (they will be captured in the mouth/throat region). According to our concept, if proposed or similar atomization methods are applied intratracheally, these droplets will have some chance to reach the destination, i.e. the surface of damaged bronchial tree. This opens an opportunity for further, more specific research on atomization of cellular colloids in the regenerative medicine in lung biology and diseases.

**Acknowledgments**

This work was supported by governmental funds for science in the years 2010-2013, Project No. NN209023339. The study is related to the activity of the European network action COST MP1106 "Smart and green interfaces - from single bubbles and drops to industrial, environmental and biomedical applications"

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