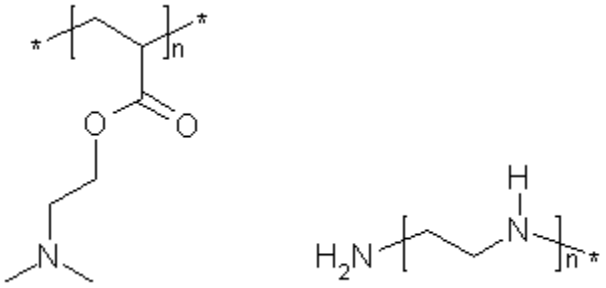
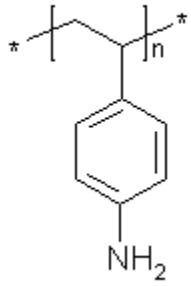
Category: **Poly-cationic Substances (Cationic Binding)** Human Health

Definition. Any polymer or substance with multiple functional groups bearing positive charges at physiologically relevant pH is a member of this class. Positive charges may be dissociable (*e.g.*, amine salts) or non-dissociable (*e.g*, quaternary ammonium cations). Such structures include polyamines, poly-quaternary ammonium, polyurea-amines, polyamide-amines and poly-guanidine compounds.

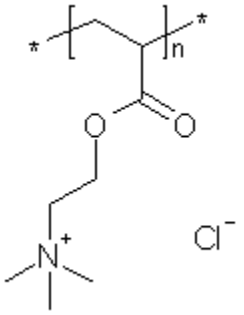
Exclusions: This category does ***not*** include compounds with formaldehyde-releasing functional groups occurring in either the polymer backbone or the substituents. These substances can include polymers with single methylene (-CH2-) groups linking amines, urea nitrogen atoms or guanidine nitrogen atoms; formaldehyde acetals; and N-hydroxymethyl and N-(alkoxy)methyl groups and their thio analogs.

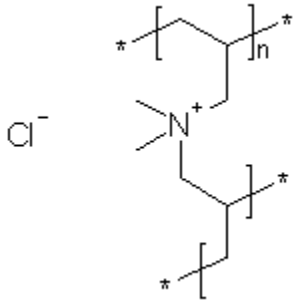
Poly-cationic category members include those with free amines:

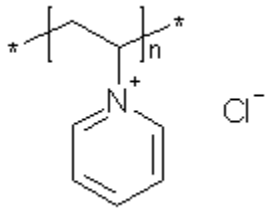




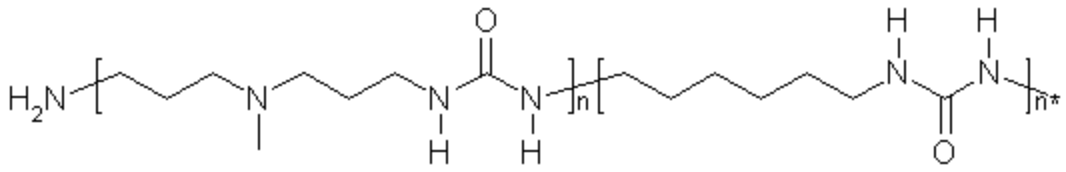
Poly-cationic category members also include substances with quaternary ammonium functional groups:

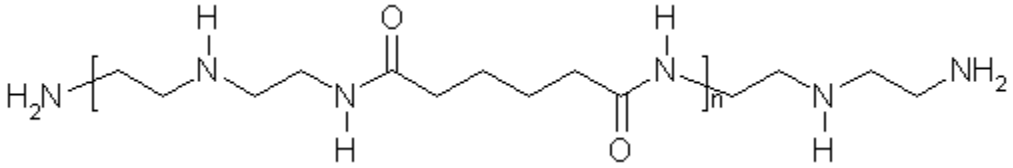




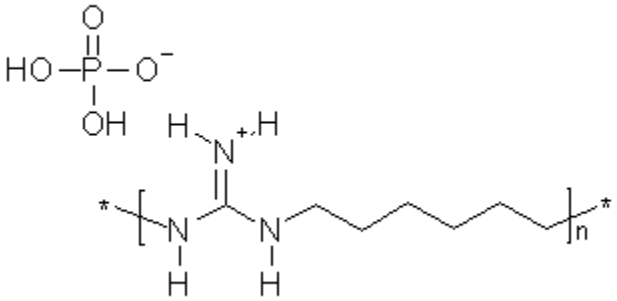


Polyureas and polyamides possessing internal or pendant amine substituents are members of this category:





Poly-guanidines and their salts are also members of this category:



Hazard Concerns.

There is concern for severe pulmonary effects from inhalation of poly-cationic polymers and substances. Inhalation of aerosols can lead to a fatal interstitial lung disease characterized by organizing pneumonia (*i.e.*, swirls of inflammatory tissue filling the alveoli and alveolar ducts) and bronchiolitis obliterans (*i.e.*, swirls or plugs of fibrous granulation tissues filling the bronchioles) in people. Studies in animal models show pulmonary inflammation and fibrosis, as well as lung function changes indicative of obstructive pulmonary disease. The mode of action appears to involve electrostatic interaction with pulmonary cell membranes resulting in disruption of lipid bilayers via permeabilization, membrane thinning and nano-scale hole formation. Effect levels *in vivo* are not well defined and cytotoxicity of class members *in vitro* is highly variable, depending on a combination of several variables including molecular weight, charge density, 3-D structure and polymer flexibility.

Supporting Data. Several outbreaks of interstitial lung disease have been attributed to inhalation of poly-cationic polymer aerosols. Textile workers in Spain and Algeria exposed to aerosols containing amine-functional polyurea and polyamide polymers during spray painting were diagnosed with organizing pneumonia and bronchiolitis obliterans (Moya et al., 1994; Ould Kadi et al., 1994). In Spain, 22 workers (including 6 who eventually died) were diagnosed by chest X-ray, CT scan and lung biopsy (Moya et al., 1994). Pulmonary disease was attributed to

inhalation of an aerosol from a specific process modification that involved a substitution of polymers. The first three cases were discovered 6 – 9 months after the polymer substitution occurred. Four cases who developed the disease in less than 5 months reported that symptoms began after one month after exposure. The factory location with the highest number of cases (n=16) had concentrations of airborne aerosol ranging from 5 to 16 mg/m^3 (mean of 10 mg/m^3). In Algeria, three female spray painters (one of whom eventually died) were admitted to the hospital in respiratory distress (Ould Kadi et al., 1994). These workers reported that respiratory symptoms began 5 to 10 months after spray painting with products from Spain started. Chest X-rays showed bilateral alveolar, interstitial infiltrates and severe effects on pulmonary function (decreased forced vital capacity [FVC] and forced expiratory volume in 1 second (FEV_1]). Two additional female workers had similar pulmonary symptoms, but were less severely affected. Chest X-ray findings and pulmonary function did not improve when the surviving women were tested more than one year later (Ould Kadi et al., 1994). Exposure to a poly-guanidine aerosol as a humidifier disinfectant resulted in several outbreaks of acute interstitial lung disease with high mortality in post-partum women and their children in South Korea (Hong et al., 2014; Kim et al., 2014a,b). The disease course began with mild respiratory symptoms that progressed rapidly to respiratory distress and respiratory failure. The median duration of cough before hospital admission in children was 21 days (Kim et al. 2014b). Pathology results from explanted and autopsy lungs of young adults showed that early stages of the lung disease were characterized by bronchiolitis and inflammatory infiltration of the alveolar septa. Bronchiolar destruction with scarring was observed at a later stage and the alveoli were remodeled by inflammation and fibrosis (Hong et al., 2014).

Experimental animal studies have generally confirmed the pulmonary findings in humans; however, only two studies were conducted using aerosol inhalation (Park et al., 2014; Nemery et al., 1988). A poly-guanidine aerosol administered to rats 6 hours/day, 5 days/week for 4 weeks resulted in interstitial lung disease characterized by destruction of bronchioles and peribronchiolar interstitium with inflammation and fibrosis (Park et al., 2014). This study employed a single exposure concentration (1.6 mg/m^3) and did not describe use of a control group. In addition, details regarding the characteristics of the aerosol (*e.g.*, particle size distribution) were not provided. Nemery et al. (1998) exposed rats to amine functional polyurea and polyamide polymer aerosols at concentrations of 50 or 250 mg/m^3 for 6 hours/day, 5 days/week for 2 weeks and described the resulting pulmonary damage (at the lowest concentration) as diffuse parenchymal injury followed by inflammatory and fibrotic sequelae. The brief summary report provided did not describe the use of control group, the strain, sex or group sizes of rats used in the experiment, the atmosphere generation method, the exposure type (*i.e.*, whole-body or nose-only), or the particle size distribution. In addition, the results of the pulmonary function tests, BALF analysis and organ weight measurements were not reported. Intra-tracheal instillation of an amine functional polyurea polymer to rats resulted in pulmonary function changes and altered blood gases 4, 8 and 12 weeks after instillation. The data suggest a mismatch of the ventilation-perfusion relationship indicative of obstructive pulmonary disease that persists for at least 3 months following exposure (Pauluhn, 2000). In hamsters given a free amine polymer or amine functional polyurea or polyamide polymers by intra-tracheal instillation, a fulminant inflammation of the lung parenchyma and diffuse alveolar damage were observed (Clottens et. al., 1997). The early response to exposure (measured at 3 and 7 days following instillation) included pulmonary edema, exudation and inflammation, with fibrosis occurring

later (at 14, 28 and 92 days after instillation). Intra-tracheal instillation of a poly-guanidine polymer in mice increased the level of inflammatory cytokines and infiltration of immune cells into the lung 7 and 14 days after instillation (Song et al. 2014). A dose-related increase in the severity of pulmonary inflammation and fibrosis was observed in this study.

There is a large body of literature related to the development of cationic polymers as vectors for therapeutic gene delivery. Many studies have investigated possible modifications to polymers in order to maintain transfection efficacy (ability to enter the cell nucleus) while reducing systemic and *in vitro* cytotoxicity (reviewed by Lv et al., 2006). Reports of systemic toxicity included effects on the blood and the lung. Intravenous (i.v.) administration of polyethylenimine (PEI) and poly-L-lysine (PLL) resulted in binding to albumin and the surface of red blood cells (RBCs) leading to cell clumping (*i.e.*, hemagglutination) and subsequent hemolysis (reviewed by Lv et al., 2006; species not reported). Poly-cationic compounds like protamine sulfate and PLL were implicated as cause of pulmonary edema in humans following i.v. administration to reverse the anticoagulant effects of heparin. A study using an isolated perfused rat lung model showed severe endothelial cell damage from these compounds that was charge mediated (*i.e.*, reversed by heparin) (Chang et al., 1987). Pulmonary endothelial disruption leading to bleeding and sanguineous lung edema was also demonstrated in Sprague-Dawley rats given PLL (>30kDa) intravenously (Isaksson et al., 2014).

Many studies have evaluated the cytotoxicity of poly-cationic polymers in mammalian cell lines (Lv et al., 2006). Fisher et al. (2003) compared the cytotoxicity of PEI, PLL, diethylaminoethyl-dextran (DEAE-DEX), poly(amidoamine) dendrimers (PAMAM), poly-(diallyl-dimethyl-ammonium chloride) (DADMAC), and poly(vinyl pyridinium bromide) (PVPBr) in L929 mouse fibroblasts (*i.e.*, MTT and LDH assays and microscopy). The relationship between molecular weight and cytotoxicity (*i.e.*, higher molecular weight compounds are more toxic) applied only to compounds of the same polymer structure (*e.g.*, 500 kDa DEAE-dextran was less toxic than 36.6 kDa PLL). The 3-D arrangement of cationic residues and flexibility of the polymer backbone determined accessibility to the cell membrane and subsequent cytotoxicity. For example, polycations characterized by a globular structure (cHSA, PAMAM) had lower cytotoxicity compared to polymers with a more linear or branched and flexible structure (PLL, PEI). The ring systems of DADMAC reduce the flexibility of the polymer and subsequent cytotoxicity. PAMAM had lower cytotoxicity because of its low MW (6.9 kDa) despite the relatively high charge monomer ratio (0.0088). DEAE-dextran had lower cytotoxicity because the number and density of cationic charges is relatively low (charge to monomer ratio of 0.00278) despite its high MW (500 kDa). The results of this study suggest cytotoxicity of poly-cationic polymers is affected by a combination of several variables (*e.g.*, size, shape, flexibility, charge density).

In vitro studies using lipid bilayers and live cell membranes are useful for evaluating the mode of action for poly-cationic polymers. These studies demonstrate that poly-cationic polymers can produce disruption of lipid bilayers, permeabilization of cell membranes, membrane thinning and nano-scale hole formation in cell membranes (Hong et al., 2006). For the polymers tested, the effect on membrane permeability was strongly dependent on number of charges on the polymer

backbone. Free amine and amine functional polyurea and polyamide polymers were cytotoxic to rat and human Type II pneumocytes and alveolar macrophages *in vitro* (Hoet et al., 1999). The observed cytotoxicity was abolished in the presence of poly-anionic compounds (sulodexide, DNA, poly-L-glutamic acid), suggesting that toxicity is related, at least in part, to surface charge on the polymers (Hoet et al., 2001).

Physical and chemical properties can be measured to inform the concern level for this class of compounds and inclusion in the category.

- If data are not available to calculate the functional group equivalent weight or the % amine nitrogen, then a test should be performed to measure the charge density in milli-Equivalents/gram.
- Data should be provided regarding the form (protonated vs. non-protonated), size (MW), pH, pKa, and shape (*i.e.*, branched, linear, globular) for each compound.

Boundaries. Polymers must be water-soluble or water-dispersible. This category contains a large and structurally diverse group of compounds; therefore, EPA is interested in test data that addresses toxicity as it relates to form (protonated vs. non-protonated), size (MW), pH, pKa, charge density and shape (*i.e.*, branched, linear, globular). Thus, this category may be modified as new information becomes available to the Agency.

General Testing Strategy[1]

Consistent with the amended Toxic Substances Control Act (TSCA), the multi-tiered testing methods below employ new approach methodologies (NAMs) to reduce the use of vertebrate animals in chemical testing. It incorporates *in chemico* characterization of the chemical substance in Tier I (particle size distribution, charge density) and structured *in vivo* testing from acute testing, sub-acute testing and sub-chronic testing in Tier II. It is recommended that any questions on the test strategy should be directed to the Agency.

Tier I – Use physical-chemical properties to characterize lung exposure/binding potential:

- Charge density in milli-Equivalents/gram or functional group equivalent weight or % amine nitrogen

- Particle Size Distribution or Aerosolized Droplet Size [*i.e.* cascade impactor, laser methods; OECD Test Guideline (TG) 110, OPPTS 830.7520, OECD Guidance Document (GD) 39]

If respirable particles or aerosols can be generated during manufacturing, processing, or any of the uses, proceed to Tier II. If not respirable, determine if Tier II testing is needed

[1] Submitters may request to use comparable test guidelines other than those listed, pending EPA's review and approval.

Tier II- *In Vivo* Studies

- Step 1: OECD Acute TG 403 (modified)** featuring rats exposed for 4 hours and observed for 2 weeks. Proceed to step 2 if LOAEC < 2000 mg/m^3.
- Step 2: 5-day inhalation study with a 14-day recovery period ** to address progression of effects (use OECD TG 412, but conduct exposure duration for at least 5 days). Proceed to step 3 if study reports a substantial decrease in the point of departure over time relative to the acute study or increase in lung burden.
- Step 3: OECD TG 412**: 28-day inhalation study in rats with a14-day recovery period

** Modifications to all above studies should include pulmonary function testing, lung burden, analysis of BALF, LDH release, blood oxygen (pO_2) content, and satellite reversibility. OECD TG 412 and OECD GD 39 should be consulted.

Furthermore, the following *in vitro* test methods for cytotoxicity and irritation can provide potentially useful information:

- ICCVAM Recommended Protocol for the BALB/c 3T3/A549 lung cells Neutral Red Uptake (NRU) Cytotoxicity Test - A Test for Basal Cytotoxicity (https://ntp.niehs.nih.gov/iccvam/docs/acutetox_docs/brd_tmer/at-tmer-appxc1-508.pdf)
- ICCVAM Recommended Protocol for the Normal Human Epidermal Keratinocyte (NHK) Neutral Red Uptake (NRU) Cytotoxicity Test - A Test for Basal Cytotoxicity (https://ntp.niehs.nih.gov/iccvam/docs/acutetox_docs/brd_tmer/at-tmer-appxc2-508.pdf)
- OECD *In vitro* Skin Irritation (Test 439) – reconstructed human epidermis test method (Note: Test 404 for skin irritation and corrosion is *in vivo*).

Supporting Data. The available studies, although limited, provide some guidance for testing of the pulmonary effects of poly-cationic polymers by inhalation exposure. Pulmonary effects were observed following 2- and 4-weeks of aerosol exposure to poly-guanidine or amine functional polyurea and polyamide polymers in rats, suggesting that a sub-acute study may be of adequate duration to detect toxicological effects (Park et al., 2014; Nemery et al., 1998). Changes in pulmonary function measurements and BALF parameters represented early and sensitive indicators of lung injury (Pauluhn, 2000; Clottens et al., 1997) after exposure to free amine and amine functional polyurea and polyamide polymers.

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Fischer, D., et al. (2003). *In vitro* cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterials 24(7): 1121-1131.

Hoet, P. H., et al. (2001). Polyanions protect against the *in vitro* pulmonary toxicity of polycationic paint components associated with the Ardystil syndrome. Toxicology and Applied Pharmacology 175(2): 184-190.

Hoet, P. H., et al. (1999). *In vitro* cytotoxicity of textile paint components linked to the "Ardystil syndrome". Toxicological Sciences : Official Journal of the Society of Toxicology 52(2): 209-216.

Hong, S., et al. (2006). Interaction of polycationic polymers with supported lipid bilayers and cells: nanoscale hole formation and enhanced membrane permeability. Bioconjugate Chemistry 17(3): 728-734.

Hong, S. B., et al. (2014). A cluster of lung injury associated with home humidifier use: clinical, radiological and pathological description of a new syndrome. Thorax 69(8): 694-702.

Isaksson, K., et al. (2014). *In vivo* toxicity and biodistribution of intraperitoneal and intravenous poly-L-lysine and poly-L-lysine/poly-L-glutamate in rats. Journal of Materials Science. Materials in medicine 25(5): 1293-1299.

Kim, H. J., et al. (2014). A cluster of lung injury cases associated with home humidifier use: an epidemiological investigation. Thorax 69(8): 703-708.

Kim, K. W., et al. (2014). Humidifier disinfectant-associated children's interstitial lung disease. American Journal of Respiratory and Critical Care Medicine 189(1): 48-56.

Lv, H., et al. (2006). Toxicity of cationic lipids and cationic polymers in gene delivery. Journal of Controlled Release : Official Journal of the Controlled Release Society 114(1): 100-109.

Moya, C., et al. (1994). Outbreak of organising pneumonia in textile printing sprayers. Collaborative Group for the Study of Toxicity in Textile Aerographic Factories. Lancet (London, England) 344(8921): 498-502.

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Park, S., et al. (2014). Humidifier disinfectant-associated interstitial lung disease in an animal model induced by polyhexamethylene guanidine aerosol. American Journal of Respiratory and Critical Care Medicine 190(6): 706-708.

Pauluhn, J. (2000). Repeated pulmonary function measurements in rats after intratracheal instillation of a polyamine textile pigment component. Inhalation Toxicology 12(7): 591-604.

Song, J. A., et al. (2014). Polyhexamethyleneguanidine phosphate induces severe lung inflammation, fibrosis, and thymic atrophy. Food and Chemical Toxicology : an international journal published for the British Industrial Biological Research Association 69: 267-275.

Zhang, H., et al. (2015). Improvement of pulmonary absorption of poorly absorbable macromolecules by hydroxypropyl-beta-cyclodextrin grafted polyethylenimine (HP-beta-CD-PEI) in rats. International Journal of Pharmaceutics 489(1-2): 294-303.

APPENDIX 1: Search Strategy

Computerized literature searches were conducted in PubMed in November 2016 to obtain studies related to the pulmonary effects of polycationic polymers. The search query string is presented in Table 1.

Table 1. Summary of detailed search strategies for Polycationic Polymers

Database Search Date	**Query String**
PubMed	
11/15/2016	((("surface properties"[mh] OR "surface charge"[tw] OR "positively charged"[tw] OR cations[mh] OR polycations[nm] OR lung[mh] OR "membrane lipids"[mh]) AND (polymers[mh]) AND (lung*[tw] OR polymer*[tw] OR bind*[tw] OR bound[tw] OR lipid*[tw] OR membrane*[tw]) AND (charge*[tw] OR cation*[tw]) AND (cytotox*[tw] OR toxic*[tw])) NOT nanoparticles[mh])

Screening methods for this search include manual screening of titles/abstracts and screening of full text articles using the eligibility criteria shown in Table 2.

Table 2. Eligibility criteria for screening of literature search results for polycationic polymers

PECO element	Evidence
Population	Humans, laboratory animals (rats, mice, hamsters, guinea pigs, dogs, non-human primates, or other inbred mammals) and mammalian cell lines
Exposure	*In vivo* (all routes), *ex vivo* (isolated perfused lung) and *in vitro*
Comparison	Any comparison (across dose, duration, or route) or no comparison (*e.g.*, case reports without controls)
Outcomes	Any examination of: • Pulmonary effects *in vivo* or *ex vivo* studies • Cytotoxicity in *in vitro* studies

The results of the literature screening for polycationic polymers are presented graphically in Figure 1. The database search results were supplemented by a review of the reference lists from relevant publications (*i.e.*, tree searching[2] yielded an additional 21 references for full text review). The *in vitro* studies that were excluded after full-text review (n=83) were focused on the transfection efficacy of polymeric gene vectors with minimal information provided on cytotoxicity. These studies did not provide useful mechanistic data for evaluation of the mode of action for pulmonary toxicity.

[2] This is also referred as backward reference searching.

Figure 1. Literature search and screening flow diagram