Dental applicability of polycationic polymers

PhD thesis

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1. Introduction

Antimicrobial polymers, also known as biocidal polymers. Within a large group of polymers, substances have growth inhibitory or killing properties against pathogenic microorganisms.

For a long time, the view prevailed that cationic polymers bound to membranes and exerted their antimicrobial activity by disorganizing them. In recent years, it has been shown that these polymers may have alternative and / or additional non-membrane targets. Based on these, polymers can be classified into two groups: membrane-damaging and non-membrane-damaging polymers. It is difficult to differentiate because in contrast to different pathogenic microorganisms, the mechanism of action of polymers may be different. Regardless of which group the polymers belong to, they must interact with the membranes. They can disorganize the membrane or reach their final intracellular target.

By forming pores in the membrane of pathogens, they increase the permeability of the cell membrane, resulting in an upset of the ion concentration and electrical potential difference between the cell plasma and the extracellular space and, at higher concentrations, destabilization of the membrane. In addition to their physical mechanism of action and their different nature from conventional antibiotics, they do not develop resistance in the classical sense. This makes them a promising target for drug research and healthcare.

Polycationic polymers can generally be characterized by two functional components, one with a positively charged cationic group and the other with a hydrophobic group. Basically, with the help of positive charges, the polymer can bind to the pathogen surface, while the hydrophobic parts are able to form channels in the membrane and disorganize the membrane. Because of these properties, the presence of both groups is essential for the effectiveness of polycationic polymers.

Primary structure of polycationic polymers

In the synthesis of polycationic polymers, the appropriate ratio and number of the two main groups (hydrophobic and cationic) are responsible for the effect. In terms of the formation of the primary structure must be determined. For cationic groups, there are several choices, including amino groups, sulfonium ions, and phosphonium ions. Amino and imino groups are most commonly used because of their ease of synthesis and wide applicability.

In addition to the polycationic property, the structure of the polymers is also important. Dendrimers are artificial macromolecules with regular structure, spatially branched, and composed of layers. Generally, monomeric units attached to the core at the center of the molecule are built up layer by layer. These layers are called generations. Core-free but tree-branched polymers are called dendrons. The functional groups at the end of the chains, the branch points, and the “cavities” within the molecule allow these molecules to be used in many fields of chemistry and science. Polycationic dendrimers and high-branched polycationic polymers are well-defined low-polydispersity substances with densely located positive charge groups on their surface, and therefore have an outstanding effect against pathogenic microorganisms.

1.1. Classical antimicrobials and resistance

Antimicrobial resistance (AMR) is one of the biggest global challenges nowadays and in the future; compromising medical advancements and our ability to treat infectious diseases. Increased antimicrobial resistance results in increased morbidity and mortality due to infectious diseases worldwide. The lack of discovery of new compounds from natural substances and new antimicrobial researches encourages the researchers to re-extract their previous studies that have been removed from routine use, such as colistin. Because the discovery of classes of new compounds is extremely costly and operates with a very low success rate, one strategy to overcome this issue may be the use of synthetic compounds with antimicrobial activity.

Currently, antimicrobial resistance is severely affecting 22 countries, with 500,000 people infected with multidrug-resistant pathogens, according to their data. O’Neill and colleagues report that by 2050, there will already be 10 million deaths a year as a result of AMR.

2. Aim of the study

The primary goal of our work was to be able to develop another substance synthesized by our research group for the treatment of denture stomatitis, in addition to the classical antimicrobial agents, up to the level of clinical use. In the present case, novelty does not only mean the creation of a hitherto unknown combination of already known substances, but also the development of a new mechanism of action and a new mode of use.

In our experiments, we would like to answer the following questions:

• What spectrofluorimetric properties PEI solutions of different molecular weights have, which other factors influence these properties?

• Can we create a polymer complex by applying PEI and nanosilver?

• Is this polymer complex in the nanosize range?

• How the active ingredient can be applied to the mucosal surface of acrylate-based dentures?

• Does the applied layer affect the stability of the denture?

• How does the spatial structure and shape of the polymer complex look like?

• What are the cytotoxic, apoptotic properties of the drug released from PEI-PLA and Ag-PEI-PLA plates?

• What is the antimicrobial effect of PEI and Ag-PEI polymer complex?

3. Materials and Methods

3.1. Synthesis of Ag-PEI-PLA polymer composite

Dissolve 1 g of PEI in 2 mL in distilled water, in a glass jar using a magnetic stirrer. In another glass vessel, dissolve 158 mg of $AgNO_3$ (100 mg of Ag) in 1 ml of distilled water with gentle stirring. After the shaking, the solutions were centrifuged for 10 minutes at 20,000g (Micromax RF, Thermo Fisher Scientific, USA). The silver nitrate solution was slowly added dropwise to the PEI solution with continuous stirring. The yellowish solution was placed on the plate of a hot plate stirrer (VELP Scientifica, Italy) set at 90°C to evaporate the water. During the process, the color of the solution changes to dark brown, and after evaporation of the water, its volume does not change, and the magnetic stirrer stops there. The temperature is then raised to 140 °C and heated for a further 4 hours until the color changes to a dark grayish brown, thick mass.

The polylactic acid was dissolved in chloroform to give a 5% w/v solution. First, 20 mg of the honey-density active complex was added to 2 mL of PLA solution and dissolved by slow shaking for 30 minutes using a laboratory shaker (IKA Works, Wilmington, NC, USA). The Ag-PEI was easily solubilized in the 5% PLA solution, which turned yellowish-brown. Subsequently, a 30-min ultrasonic treatment was performed (45 kHz, 100 W; Emmi 12HC, EMAG, Germany). The practically colorless supernatant was gained with a 2 min, 2000 rpm centrifugation (BW41-BR230, Qualitron Inc., Korea), and the aggregated pellet was discarded. The resulting supernatant is suitable for coating the surface of dentures.

3.1.1. Preparation of PEI solutions

In the preparation of PEI solutions, the factory products sold by Sigma-Aldrich were used in all cases 800 MW, 2000 MW, 25 000 MW, 750 000 MW. After adding an appropriate amount of distilled water, the measured material was stirred with a magnetic stirrer at room temperature for 1 hour. The resulting solution was then centrifuged at 20 000 g for 10 minutes. No filtration was

applied because without filtration we have obtained a purer preparation based on our DLS studies.

3.2. Spectrofluorimetry

3.2.1. Fluorescence measurement of PEI dilution series

Fluorescence intensity measurements were performed in each case with a Hitachi F-4500 FL spectrophotometer. For PEI solutions, a series of solutions (0.625–1.25–2.5–5–10 mg / mL (w/v)) were prepared from four concentrated PEIs of different molecular weights (0.8–2–25–750kPEI). The pH of the solutions was 10.6 for all samples. PEI was dissolved in Eppendorf tubes in distilled water with gentle shaking for 4 hours. The resulting solutions were centrifuged at 20,000 g for 10 min and the supernatant was used for measurements.

3.2.2. The measurement of pH and fluorescent of PEI dilution series

The pH was measured with the pH meter, Hanna Piccolo Plus P9565-1EA from Sigma-Aldrich. The pH determination of PEI solutions in DW (0.8–2–25–750 kPEI MW) was carried out. The initial concentration (10 mg/mL) was used in the case of every molecular weight. The dissolution process was performed as previously described. The measurement concentration (10 mg/mL) was the same for all molecular weights. For 25kPEI, fluorescence was also measured at different pH values. The different pH values were adjusted with hydrochloric acid. Each pH measurements were carried out in different samples supplemented until the same volume, therefore the possible measurement deviation derived from dilution was eliminated.

3.2.3. The Measurement of Absorbance of PEI dilution series

Absorbance was measured with a DeNovix DS-11 FX drop spectrophotometer. Using the integrated UV-Vis software (220-750 nm wavelength) and from 3μL samples. Calibration was performed with distilled water in all cases. During the measurements, the absorbance of 25kPEI solutions of different concentrations (10–5–2.5–1.25–0.625–0.3175 mg / mL) was determined. In addition, it was measured at the same concentration (1 w/v%) for PEI solutions of different

molecular weights. PEI was dissolved in Eppendorf tubes in distilled water with gentle shaking for 4 hours. The dissolution process was performed as previously described.

3.3. Atomic Force Microscopy of the Ag-PEI-PLA composite

Atomic force microscopy imaging was performed with a Dimension Icon (Bruker, Palaiseau, France) microscope on the surface of Ag-PEI-PLA coating. Measurements were performed in the so-called “tapping” mode at room temperature (~ 22-24 ° C) and ~ 50% humidity. The TESPA-V2 (Bruker, Palaiseau, France) type needle with a spring constant of about 42 N/m and a resonance frequency of about 320 kHz proved to be optimal for our measurements.

3.4. Dynamic Light Scattering (DLS)

The Zetasizer Nano S 90 (Malvern Instruments Ltd., Malvern, UK) dynamic light scattering (DLS) was used to determine the size distribution. Z-average values were used for the primary evaluation of measurements. Dissolving PEI and Ag-PEI polymer complex in distilled water was measured at 25 ° C three times after one minute of rest.

3.5. Micro-Computed Tomography of the Ag-PEI-PLA Composite

The Ag-PEI-PLA polymer composite film was scanned using a micro-CT instrument (SkyScan 1172 micro-CT, Bruker, Kontich, Belgium). The measurement parameters were as follows: 1.91 μm isometric cube voxel with no filter; 40 kV tube voltage and 200 μA tube current values with 0.5 ° rotation step. The raw images were reconstructed using CTAn and CTVol (Bruker, Kontich, Belgium) software.

3.6. Release measurement with gravimetric method

Eight glass microscope slides, each with a surface area of fourteen square centimeters (27 x 76 mm), were coated with Ag-PEI-PLA polymer composite dissolved in 1 ml of chloroform. The other eight slides were coated with 5% PLA dissolved in 1 ml of chloroform. After evaporation

of the solvent, slides were placed in distilled water for 8 days. On days 1., 2., 3., 4., 5., 8., after air drying, weight loss was measured using an analytical balance.

3.7. Impedimetry

Cultures of the HGEP cell line (CELLnTEC, Bern, Swiss) were maintained in CnT-24.S medium (CELLnTEC, Bern, Swiss) containing 1% L-glutamine and supplemented with 1% penicillin-streptomycin (Lonza Group Ltd., Switzerland).

The effects of Ag-PEI-PLA and PEI-PLA supernatants on cytotoxicity and cell adhesion were investigated using an xCELLigence RTCA SP (ACEA Biosciences Inc., San Diego, USA) impedance-based system. Real-time assays were performed with 96-well E-plates (ACEA Biosciences Inc., San Diego, USA) in a 15 kHz AC system at a sampling rate of 20 seconds. The measuring electrodes were not covered with peptides before the measurements, so-called “nude” electrodes were used to prevent unwanted side effects.

3.7.1. Cytotoxicity Assay

First, the proliferation of untreated HGEP cultures was examined by impedimetry using 96-well E-plates. After 24 h, the cultures were treated with the 1- or 5-day PEI-PLA or Ag-PEI-PLA membrane extracts (dilutions: 1000x, 100x, 10x, and 1x) for 168 h.

3.7.2. Cell Adhesion Assay

First, the impedance value of the medium was determined as a baseline and absolute control value. Second, the HGEP cells were loaded together with extracts of PEI-PLA or Ag-PEI-PLA (dilutions: 1000x, 100x, 10x, and 1x) prepared by soaking the composite membrane in cell culture medium for 1 or 5 days. The follow-up time was 12 h.

3.7.3. Apoptosis Measurement

Apoptotic HGEP cells were detected 24 hours after the addition of Ag-PEI-PLA or PEI-PLA supernatant. Two different apoptosis assays were used for this purpose. The first was performed by a computer-based morphometric analysis and the second by Annexin V staining, which is one of the most commonly used methods.

The morphometric evaluation by computer method was based on the analysis of light microscopic serial images (obj .: 20x; Axio Observer A1; Carl Zeiss Microscopy GmbH., Jena, Germany) and Fiji ImageJ software.

3.8. Microbiology assays

The minimum inhibitory concentration (MIC) for their PEI and Ag-PEI dissolution series was determined by broth microdilution assay incubated overnight. In both cases, the samples were incubated for 24 hours. Two types of diffusion techniques were used in the measurements, one is well diffusion technique and the other is the disk diffusion test. E. faecalis (ATCC29212) on Müller-Hinton agar medium with 4% glucose added to C. albicans (ATCC66027) and S. mutans (ATCC35668) on Mitis-Salivarius agar medium at 37 ° C and 5% CO2 have been bred.

3.9. Statistical Analysis

The delta CI (ΔCI) and slope values were calculated by the integrated software (RTCA 1.2, Roche Applied Science, Indianapolis, IN, USA) of xCELLigence SP System. For further analysis of the data, Origin Pro 8.0 (OriginLab Corporation, Northampton, MA, USA) was used. Data shown represent mathematical averages of three parallels and ±SD values. Statistical analysis of data was done by the application of ANOVA of Origin Pro 8.0. Significance levels are indicated showing each result as follows: x: $P < 0.05$; y: $P < 0.01$; z: $P < 0.001$.

4. Results

4.1. Characterization of Polyethyleneimine (PEI)

4.1.1. Spectrofluorimetry

4.1.1.1. Fluorescence measurement of PEI dilution series

The fluorescence of PEI dilution series (0.625–1.25–2.5–5–10 mg/mL (w/v)) were measured in different molecular weights (0.8–2–25–750 kPEI). In every case, the increment of fluorescence was linear and could be observed with increasing concentrations. In the case of 0.8 kPEI and 2 kPEI, the count rate increased slightly while the count rates of 25 kPEI and 750 kPEI increased at higher rate. Based on our results, we can state that the linear correlation coefficients the relationship is strong (800 MW PEI = 0.9634; 2000 MW PEI = 0.9965; 25,000 MW PEI = 0.9983; 750,000 MW PEI = 0.9979). It was detected that the increase of molar concentration results in a non-linear fluorescent increment. The minimum detectable value (LOD) for PEI solutions of different molecular weights: 0.8 kPEI 0.625 mg/mL (measured); 2 kPEI 0.625 mg/mL (measured); 25 kPEI 0.229 mg/mL (extrapolated from data); 750 kPEI 0.220 mg/mL (extrapolated from data).

4.1.1.2. The measurement of pH and fluorescent of PEI dilution series

The pH of PEI solutions with the same concentration (1%) but different molecular weights (0.8–2–25–750 kPEI) was the same (pH ≈10.6). At lower pH, but at the same concentration, the fluorescence intensity was higher.

4.1.1.3. The measurement of Absorbance of PEI dilution series

In case of PEI dilution series (3.125–6.25–12.5–25–50–100 mg/mL (w/v)) the maximum value of absorbance was at 220 nm in each solution. The increment in absorbance at 220 nm is directly proportional to change in concentration. There was no difference in the absorbance spectra at the same concentration (1%) of the different molecular weights of (0.8–2–25–750k) PEI.

4.1.2. Dynamic Light Scattering (DLS)

The PEI was dissolved in DW and the concentrations were 10mg/mL and 1 mg/mL. At higher concentration, the Z-Average: 9,658nm, PDI: 0,224, with lower concentration the Z-Average: 11,07 nm, PDI: 0,508. In terms of intensity values: Pk1: 7,784nm, Pk2: 30,64 nm at 10 mg/mL; Pk1: 7,075 nm, Pk2: 42,86 nm, Pk3: 525,6 nm at 1 mg/mL. The number distributions shows at 10 mg/mL Pk1: 6,531 nm, Pk2: 23,4 nm; at 1 mg/mL Pk1: 6,512 nm

4.2. Characterization of Ag-PEI-PLA

4.2.1. Atomic Force Microscopy of the Ag-PEI-PLA Composite

Atomic force microscopic topography images show that the diameter of the spherical-shaped surface structures is in the range of 0.5–4.0 μm.

4.2.2. Dynamic Light Scattering (DLS)

The Ag-PEI was dissolved in DW and the concentrations were 10mg/mL and 1 mg/mL. In the case of higher concentration the Z-Ave: 45,69 nm a PDI: 0,153, with lower concentration the Z-Ave: 49,71 a PDI: 0,205. The intensity weighted distribution shows: Pk1: 18,42 nm, Pk2: 62,43 nm, Pk3: 3,894 nm; 1 mg/mL Pk1: 19,96 nm, Pk2: 69,18 nm, Pk3: 188,3 nm. The number distributions show 10 mg/mL: Pk1: 6,531 nm, Pk2: 23,4 nm; 1 mg/mL: Pk1: 6,512 nm.

Examination of the size distribution of the particles dissolved from the PEI-PLA and Ag-PEI-PLA films shows a significant difference compared to the control medium. Over time, the size of the dissolved particles decreases (Ag-PEI-PLA: D1, 7 nm; D5, 6 nm; D10, 4 nm). In each measurement, the size of the particles dissolved from the silver-containing composite was larger.

4.2.3. Micro-Computed Tomography of the Ag-PEI-PLA Composite

During the examination of the sample, at the maximum magnification of the micro-CT device we use, a kind of granular structure can be recognized. This structure refers to aggregation during polymer synthesis.

4.2.4. Release measurement with gravimetric method

The average weights of PLA and Ag-PEI-PLA films applied to microscopic slides were 57.58 mg and 58.76 mg, respectively. During the 8-day measurement, weight loss was observed for both types of film. Overall, on day 8, the weight loss of Ag-PEI-PLA-containing composite films (33%) was significantly greater than that of PLA films (11%).

4.3. Comparison of the PEI and Ag-PEI

4.3.1. Impedimetry

4.3.1.1. Cytotoxicity

In the case of 1-day samples, both substances were showed a cytotoxic effect on HGEP cells. The cytotoxic sensibility of the HGEP cells were proved greater in case of PEI-PLA D1 extracts (100x–1x) than the Ag-PEI-PLA extracts (10x–1x). For the day 5 extracts, the cytotoxic characters of both types of extracts were still detectable; however, the strength of toxicity in the PEI-PLA extract was similar to the Ag-PEI-PLA extract.

4.3.1.2. Cell adhesion

Comparison of the adhesion blocker abilities have demonstrated that both the PEI-PLA and Ag-PEI-PLA day 1 extracts had a significant blocking effect (dilution ranges: 1x–10x). Analysis of the results shows that the PEI-PLA preparation clearly inhibited adhesion greater than the Ag-PEI-PLA preparation.

4.3.1.3. Apoptosis

Elevated numbers of apoptotic cells were found in both the 1-day and 5-day PEI-PLA and Ag-PEI-PLA samples in both the morphometric analysis and the Annexin V assay. Comparing the results, it can be concluded that from the PEI-PLA and Ag-PEI-PLA extracts indicated PEI alone

had the strongest and widest range (100x–10x) effect, while in Ag-PEI, only 10x dilutions were effective.

4.3.2. Antimicrobial assays

Minimal inhibitory concentration values according to the microdilution assay were 1.25 mg/mL (0.125 %) at PEI and 2.5 mg/mL (0.25 %) for the three tested microorganisms. Both drugs have increased the diameters of the inhibitory zones in a concentration-dependent manner in well-diffusion technique. However, out of the three investigated microorganisms, in case of the *C. albicans* have formed consequently the highest zone of inhibition at the same substance concentrations.

5. Conclusion

- The intrinsic fluorescence of PEI was clearly defined with the help of spectrofluorimetric measurements. The intensity of fluorescence was in linear correlation with increasing concentrations, which intensity was smaller in 0,8kPEI and 2kPEI samples and greater in case of 25kPEI and 750kPEI. This correlation was not linear regarding molar concentrations. The intensity of fluorescence has increased significantly by decreasing the pH of the solution at a given concentration. The absorbance of the solvents only depended on the concentration but not on the molecular mass.

- The Ag-PEI polymer-complex and the Ag-PEI-PLA polymer-composite were successfully synthesized.

- The DLS measurements have proved that the Ag and Ag-PEI molecule sizes were in nanometer range. The hydrodynamic diameters of released molecules from the Ag- PEI-PLA membranes were D1: 7 nm, D5: 6 nm, D10: 4 nm.

- The application of the antimicrobial Ag-PEI-PLA polymer-composite layer could be easily carried out chairside by the dentist. The applied layer does not influence the stability of the denture.

- Based on the AFM and micro-CT recordings, the Ag-PEI polymer-complex had a ball-like shape.

- According to the release results of the antimicrobial layers from plates, the weight loss was higher from Ag-PEI-PLA than from PLA membranes. It can be concluded that the active agent could be released from the antimicrobial layer.

- The measurements of cytotoxicity, apoptosis and cell adhesion showed that the Ag-PEI-PLA had lower levels of cytotoxicity, apoptotic and adhesion inhibiting capacity than the PEI-PLA.

- The antimicrobial measurement results showed that both PEI and Ag-PEI were effective against the studied microorganisms. The highest effectiveness was detected against C. albicans. It can be concluded that it can be effectively applied in the treatment of denture stomatitis.

- The cytotoxicity of Ag-PEI was weaker than that of the PEI. At the same time, Ag-PEI samples had a slightly lower antimicrobial capacity. It can be concluded that the Ag-PEI is far less cytotoxic and slightly less weak regarding the antimicrobial effectiveness.

6. Publications

Géczi Z, Kispélyi B, Pál K, Hermann P: Baktérium- és gombaölő polimerek a fogászatban Egy új, hatékony antibakteriális, antifungális, kationos polimer, a polietilénimin fogorvosi felhasználásának lehetőségei Fogorv Szle (2016) 109 : 56-60.

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IF: 2,233

Tóth V, Hermann P, Végh D, Zelles T, **Géczi Z**. (2019) Study of the Intrinsic Fluorescence of a Highly Branched Cationic Dendrimer, Poly(Ethyleneimine) (PEI). Molecules, 24: 3690.

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