Toxicity and dose-response of intraabdominally administered α-poly-l-lysine and poly-l-glutamate for postoperative adhesion protection.

Karolin Isaksson, Daniel Åkerberg, Roland Andersson, Bobby Tingstedt

Department of Surgery, University Hospital of Lund, Sweden

Correspondence to;

Bobby Tingstedt, MD, PhD

Department of Surgery

Lund University Hospital

S-221 85 Lund Sweden

Email: Bobby.tingstedt@med.lu.se

Abstract

Background/Aims

Two differently charged polypeptides, poly-l-lysine and poly-l-glutamate have previously been shown to reduce postoperative intraabdominal adhesions. This study aims to investigate possible toxic effects and establish a lowest effective anti-adhesive dose.

Methods

152 mice were investigated with a well known adhesion model and given different concentrations of the two differently charged polypeptides as well as only the cationic poly-l-lysine.

Results

A probable toxic level of poly-l-lysine intraperitoneally could be established for the first time (40 mg/kg) and the lowest significant concentration of poly-l-lysine and poly-l-glutamate for antiadhesive purpose (1.6 mg/kg).

Conclusion

The gap between possible toxicity level of poly-l-lysine and the lowest efficient antiadhesive dose is probably too narrow and the shape and charge of poly-l-lysine warrants continuous research for another polycation in the concept of differently charged polypeptides as antiadhesive agents.

Key Words: postoperative adhesions, polypeptides, dose-response, toxicity

Introduction

Intraabdominal adhesions develop in almost every abdomen that has been operated upon. Abdominal pain, female infertility and intestinal obstruction are the most frequently reported symptoms related to adhesion formation[1]. Postoperative intraabdominal adhesions are a benign condition, but carry a high morbidity for those affected. Adhesions represent the cause for almost 75% of small bowel obstruction[2], 20-40% of reported female infertility[3] and greatly increase the risk of inadvertent small intestine damage at subsequent operations[4]. Adhesions is also costly for the society with a reported yearly cost of 6.3 million €/ million inhabitants.[5].

Various bioactive materials have been used for treatment both experimentally and clinically[6], including hyaluronic acid and its derivates[7], soluble polysaccharides[8] and phospholipids[9]. We have previously shown that differently charged bioactive polypeptides, poly-L-lysine and poly-L-glutamate, significantly prevent abdominal adhesion formation in various experimental settings[10-12].

Poly-L-lysine is a strong cation and poly-L-glutamate is an anion. Together they form a non-toxic matrix which reduces postoperative abdominal adhesions[13]. However, poly-L-lysine is, due to its strong negative electrostatic charge, reported to carry side effects, such as direct cytotoxicity[14], apoptosis[15], membrane disruption[16] and ion-channel disturbances[17, 18] when used as single drug in vivo or in vitro [19, 20]. Poly-l-glutamate does not carry this side effect with its anionic charge. The aim of this study was to elucidate the possible side effects and toxic symptoms of poly-L-lysine in mouse and establish a dose where these symptoms would not occur.

Material and Methods.

Animals

152 NMRI mice weighing about 25-30 g were used. The animals were kept under standardized conditions, 22 degrees Celsius and 12-hour daylight. They had free access to pellets and tap water. The study was conducted with approval of the local ethical committee at the University of Lund.

Chemicals

An osmotic balanced (2.54 w% glycerol) aqueous solution of poly-L-lysine hydrochloride, (PL) and poly-L-glutamate, (PG) in different concentrations were freshly prepared on the day of the experiment and stored in the refrigerator until used. Poly-L-lysine is a positively charged amino acid polymer. In this experiment we used a poly-L-lysine with a size approximately above 300 nm and molecular weight > 30 kDa. The molecular weight of poly-L-glutamate was 15-50 kDa. All chemicals and liquids were purchased from Sigma-Aldrich (St Louis, Mo, USA)

Model

Anaesthesia was induced by ketamine 60 mg/kg (Ketalar, Pfizer, NY, USA) and xylazine 16 mg/kg (Rompun Vet, Bayer AB, Gothenburg, Sweden) by an intramuscular injection. After disinfection, a 25mm long midline laparotomy was performed. Both peritoneal surfaces of the lateral abdominal wall were exposed; and 15mm long incisions on each side were performed at the same distance from the midline, also involving the muscles. The wounds were immediately closed with 2x4 single sutures placed at equal distances, using 5.0 poly-propylene thread (Prolene; Ethicon, Johnson & Johnson, Somerville, New Jersey, USA) equipped with a cutting needle. The midline laparotomy was closed in two layers with a

continuous 5.0 poly-propylene suture. After one week, a time interval chosen to match our previous studies, an overdose of anesthesia was administered and the abdomen was opened through a U shaped incision with its base to the right. The lengths of the individual abdominal adhesions attached to the incisions were measured as well as the lengths of the abdominal incisions themselves. A calliper was used for measurement and data was expressed in percent of the incision covered by adhesions[21] . All animals were treated with buprenorphine. Animals that showed signs of non-survival were given an overdose of anesthesia.

Experimental design

The animals were randomly divided into the various experimental groups according to table 1. The experiment was divided in two different phases. In the first phase an evaluation of the post-operative anti-adhesive effect of PL and PG was performed based on different concentrations. Table 1 summarizes the experimental design and group division where the various concentrations and volumes used also are presented.

At the end of each operation the treatment substances were installed intraabdominally upon closure of the abdominal wound, first PL and then 10-15 seconds later PG with corresponding volumes and doses (volumes and concentration shown in table 1). All control groups had sodium chloride solution, (0.9 %) similarly installed at the end of the operation in different volumes according to table 1.

Groups, 8-11, where decreasing volume and local application was investigated the same model was applied to their abdominal walls but the liquids were applied with a pipette on top of the abdominal wounds in the same time fashion as above. Animals were weighed before procedure and prior to evaluation.

In the second phase only PL was used to establish a level where toxic symptoms were detectable. The starting dose/concentration of PL was the lowest concentration with intact

antiadhesive effect seen in the first phase. PL was installed intraperitoneally at the end of the operation. Animals were weighed before procedure and prior to evaluation.

Statistical analyses

Mann Whitney U test were used to compare adhesions. Groups were compared using Kruskal Wallis test. All data are expressed as means and standard error of the mean (SEM). Differences were considered statistically significant at a p-value of <0.05. SPSS v 17.0 (SPSS Inc, Chicago, Illinois).

Results

Poly-L-lysine and poly-L-glutamate in decreasing doses

The dose PL+PG, (0.5%), used in former experiments [10] significantly reduced postoperative adhesions ($p<0.001$) and served as standard control together with the sodium chloride control group. When decreasing the dose by decreasing the concentration of poly-L-lysine, the antiadhesive effect also decreased ($p=0.05$) as compared to the standard dose of 0.5% (figure 1). All but the lowest dose (0.001% or 0.4 mg/kg) significantly reduced the postoperative adhesions compared to NaCl controls (table 2).

When lowering the dose by reducing the installed volume and applying the substances locally on top of the wounds the same pattern was seen with initially significant reduction ($p=0.001$) of abdominal adhesions and decreasing effect with decreasing volume/dose (figure 2 and table 3). In the lowest volume-dose, 4 mg/kg, however, no significant reduction of adhesions was seen as was evident in the animals who received 4 mg/kg in normal volume but lower concentration.

Poly-L-lysine alone

In the second phase of the experiment where only poly-L-lysine was used, the mice who received the lower doses (groups 12 and 13) all faired well. In group 14 half of the animals died within 24 hours. Two showed signs of distress and were prematurely sacrificed after 6 hours, the remaining four were prematurely sacrificed due to inadequate recovery after 24 hours but without obvious signs of distress (Table 4). The diseased animals all showed signs of convulsions. The other half of the animals were all well. Intraperitoneal single dose poly-l-lysine with higher concentration were not administered due to the deaths at this stage. An approximated LD50 dose (Lethal dose 50%) of intraperitoneal poly-L-lysine could therefore be set to 40 mg/kg in mice. There was no weight loss in any of the surviving animals. In this part of the study, no significant antiadhesive effect seen in any group as compared to controls.

Discussion

Polypeptides are being used widely in biologic research and encountered in different settings such as coating of implants, gene vectors, chemotherapeutic drug carriers and antimicrobial and antineoplastic research [22-27]. The polypeptides poly-α-glutamate, poly-l-α-lysine, poly-γ-glutamate and poly-ε-lysine have been stated to be water soluble, biodegradable, edible and non-toxic towards humans and environment and therefore excellent for biotechnological and biomedical applications[28]. We have previously shown a new field of use installing two differently charged polypeptides intraabdominal to prevent postoperative adhesions, bleeding and assisting in intestinal healing[10-12]. This concept with bioactive polypeptides has been without toxic side effects which is crucial in a condition, which although vast and costly as described above, is benign.

However, reports of severe toxicity in vitro and in vivo using cationic polymers for gene delivery and graft coating[16, 20, 25, 29, 30] evoked this study since the dose used in our previous experiments is to be considered high (200 mg/kg). The toxicity is owed to direct

cytotoxicity and apoptosis mediated via cell membrane disruption in high doses[15]. Lowering the concentration of the poly-l-lysine seems to evoke an inflammatory reaction and necrosis mediated via TNF-α [31]. In even lower concentration the toxicity lies intracellularly with disruption of ion channels[17, 18]. The poly-l-lysine is distributed on the cell surface and is able to migrate through the bilipid layers [32] and this quality is used in gene vector biology and formation of nanoscale holes inducing enhanced exchange of materials across the cell membrane[33]. Older studies have previously showed a toxic effect measured as (LD 50) when administered intravenously at the dose of 2 mg/kg [34]. The toxic effects was at this time explained with hemagglutination and hemolysis, later verified by Moreau et al due to the electrostatic interactions between the strong poly-l-lysine cation and negatively charged blood cells and thereby inducing lysis[20].

In our experiments mixing and using the combination of polycations (poly-l-α-lysine) and polyanions (poly-l-glutamate) demonstrated no toxic effects. This is a phenomenon well known and reported by other authors demonstrating discontinued toxicity of polycations when administered in certain sequences as regards to other ionic substances[35]. As our previous dose of 200 mg/kg intrabdominally is high compared to the reported toxic intravenous dose of 2 mg/kg, we tried to establish a dose where toxic symptoms occurred intrabdominally, something that has never been shown before and crucial to the continued usage of polypeptides as antiadhesive agents.

In the second phase half of the animals showed symptoms of toxicity at the dose of 40 mg/kg and were prematurely sacrificed. Evaluation of these animals showed no obvious macroscopic signs of inflammation or bleeding.

It is possible to state that a LD50 level is approximately 40 mg/kg in mice, higher than the intravenously LD 50 of 2 mg/kg. All animals that received lower doses than 40 mg/kg faired well, but there was no anti-adhesive effect noted as expected.

The first part of this experiment exhibited an initially significant decrease of postoperative adhesions, but the effect was decreasing accompanying the decreasing doses of the polypeptide complex regardless of whether the concentration or volumes were lowered. Finally the effect was eradicated in the lowest doses.

This further supports our earlier stated hypothesis that differently charged polypeptides reduces postoperative adhesions via electrostatic interaction and the formation of a matrix. However, the interval between the lowest dose of antiadhesive effect, 1.6 mg/kg and the LD50 dose of 40 mg/kg is probably is too narrow. Therefore the poly-l-lysine must be altered or replaced as the polycation in this concept.

Literature states that the toxicity of polypeptides and polycations in particular is not only dose dependent but also connected to molecular weight and cationic charge density[14, 19].

The poly-l-lysine used in our experiments is called the common alpha-form; poly-l-α-lysine. It is shaped as a helix and carries its charge on longer side chains than many other polylysines do[36]. These two properties probably cause the high direct cytotoxicty of poly-l-α-lysine. When in contact with the cell membranes the poly-l-α-lysine helix is elongated and connected with the membrane for a long distance enabling the long charged side arms to disturb the membrane integrity[37]. An altered polylysine with lower charge or spatially altered would therefore probably be a better alternative.

In summary, we have stated a probable toxic level for intraperitoneal administered poly-l-lysine in mice. The interval between toxicity and effect is however too narrow and we will therefore search for another polycation to replace poly-l-α-lysine in our continuing research of the postoperative antiadhesive effects of bioactive polypeptides.

Table 1. **Experimental design**

Group	Animals	Treatment	Concentration (mg/ml)	Volume (ml)	Dose mg/kg)
Phase 1					
1	10	PL+PG	5,0	1,0	200
2	9	PL+PG	1,0	1,0	40
3	10	PL+PG	0,5	1,0	20
4	10	PL+PG	0,1	1,0	4
5	9	PL+PG	0,04	1,0	1,6
6	10	PL+PG	0.01	1,0	0,4
7	10	Control (NaCl)		2,0	
8	10	PL+PG	5,0	0,1	20
9	10	Local application PL+PG	5,0	0,04	8
10	10	Local application PL+PG	1,0	0,02	0,8
11	10	Control (Nacl)		0,08	
Phase 2					
12	12	PL	0,1	1,0	4
13	12	PL	0,5	1,0	20
14	12	PL	1,0	1,0	40
15	6	Control (Nacl)		1,0	

Experimental design. PL; poly-l-lysine, PG; poly-l-glutamate.

Volumes in the first part are per each substance therefore volumes of treatment substances PL+PG should be doubled.

Table 2. **Significance of adhesion reduction in intraabdominal administration of PL/PG**

Concentration (mg/ml)	Compared with Standard dose	Compared with NaCl	Dose (mg/kg)
1.0	0,043	0,003	40
0.5	0,218	0,001	20
0.1	**0,043**	0,001	4
0.04	**0,013**	0,01	1,6
0.01	**0.001**	0.16	0.4

All groups received the same volume. P-values of decreasing concentrations of poly-l-lysine compared with standard concentration of 0.5% (200 mg/kg) and NaCl controls. Results in bold represents significantly worse results than compared with standard dose.

Table 3. **Significance of adhesion reduction in topical installation of PL/PG**

Concentration (mg/ml)	Volume (ml)	Compared with Standard dose	Compared with NaCl	Dose (mg/kg)
5.0	0,1	0,105	0,001	20
5.0	0,04	0,631	0,002	8
1.0	0,02	**0,004**	0,280	0.8

P-values of decreasing volumes and local application of poly-l-lysine and poly-l-glutamate compared with standard concentration of 0.5% (200 mg/kg) and NaCl controls. Results in bold represents significantly worse results than compared with standard dose.

Table 4. **Outcome of the animals in Phase II**

Group	Animals	Treatment	Concentration (mg/ml)	Volume (ml)	Dose (mg/kg)	Survival
12	12	PL	0,1	1,0	4	12
13	12	PL	0,5	1,0	20	12
14	12	PL	1,0	1,0	40	6/12
15	6	NaCl	9,0	1,0		12

Outcome of singledose poly-l-lysine intraabdominally.

Figure 1. **Results of adhesion reduction when altering the concentration of PL/PG**

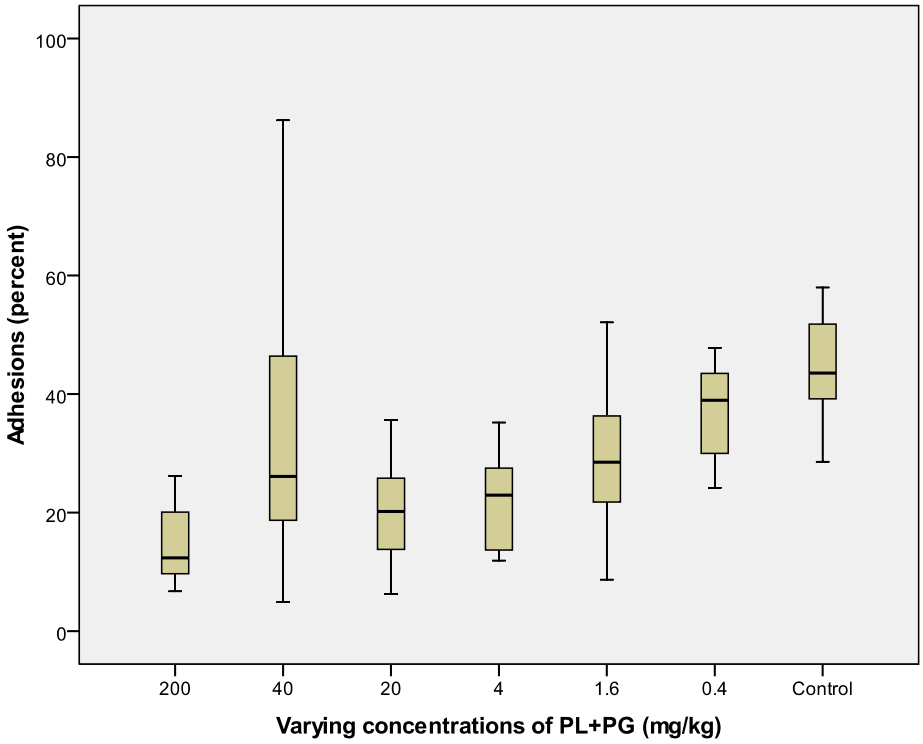
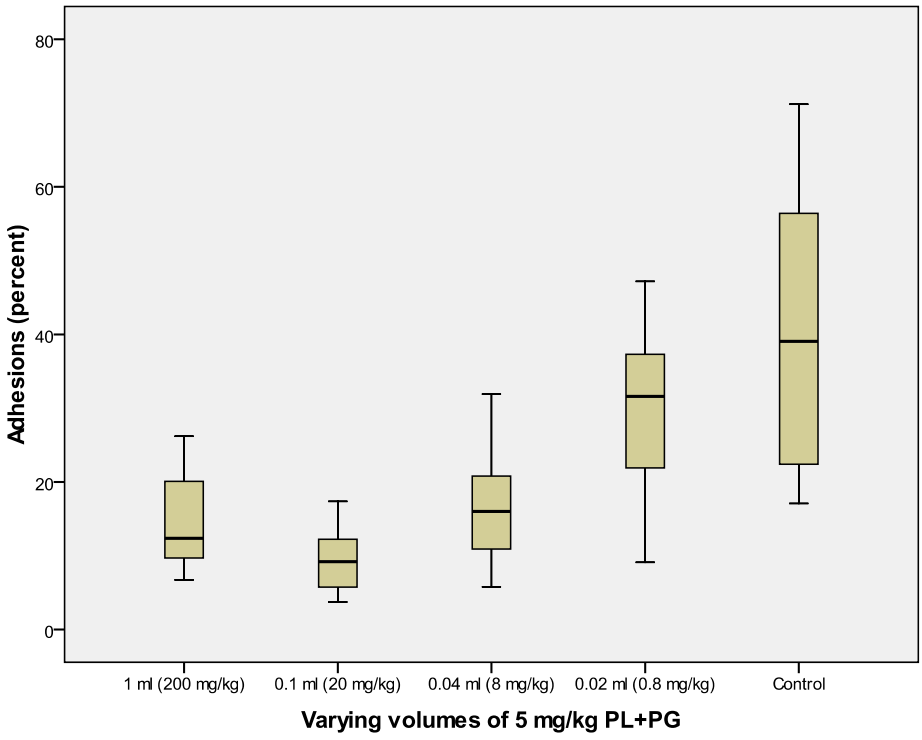


Figure 2. **Results of adhesion reduction when altering the volume of PL/PG**



References

1. Ellis H, Moran BJ, Thompson JN, Parker MC, Wilson MS, Menzies D, McGuire A, Lower AM, Hawthorn RJ, O'Brien F, Buchan S, Crowe AM: Adhesion-related hospital readmissions after abdominal and pelvic surgery: retrospective cohort study. Lancet 1999; 353: 1476-80.
2. Menzies D: Postoperative adhesions: their treatment and relevance in clinical practice. Ann R Coll Surg Engl 1993; 72: 147-53.
3. Monk BJ, Berman ML, Monitz FJ: Adhesions after extensive gynecological surgery; clinical significance, etiology and prevention. Am J Obstet Gynecol 1994; 170: 1396-403.
4. Krabben AAvd, Dijkstra FR, Nieuwenhuijzen M, Reijnen MM, Schaapveld M: Morbidity and mortality of inadvertent enterotomy during adhesiotomy. Br J Surg 2000; 87: 467-471.
5. Tingstedt B, Isaksson J, Andersson R: Long-term follow-up and cost analysis following surgery for small bowel obstruction caused by intra-abdominal adhesions. Br J Surg 2007; 94: 743-748.
6. Tingstedt B, Isaksson K, Andersson E, Andersson R: Prevention of abdominal adhesions - present state and what´s beyond the horizon? Eur Surg Res 2007; 39: 259-268.
7. Johns DB, Rodgers KE, Donahue WD, Kiorpes TC, diZerega GS: Reduction of adhesion formation by postoperative administration of ionically cross-linked hyaluronic acid. Fertil Steril 1997; 68: 37-42.
8. DiZerega GS, Verco SJ, Young P, Kettel M, Kobak WA, Martin D, Sanfilippo J, Peers EM, Scrimgeour A, Brown CB: A randomized, controlled pilot study of the

safety and efficacy of 4% icodextrin solution in the reduction of adhesions following laparoscopic gynaecological surgery. Hum Reprod 2002; 17: 1031-1038.

9. Muller SA, Treutner KH, Tietze L, Anurov M, Titkova S, Polivoda M, Oettinger AP, Schumpelick V: Efficacy of adhesion prevention and impact on wound healing of intraperitoneal phospholipids. J Surg Res 2001; 96: 68-74.

10. Nehéz L, Tingstedt B, Vödrös D, Axelsson J, Lindman B, Andersson R: Novel treatment in peritoneal adhesion prevention: protection by polypeptides. Scand J Gastroenterol 2006; 41: 1110-7.

11. Tingstedt B, Nehez L, Axelsson J, Lindman B, Andersson R: Increasing anastomosis safety and preventing abdominal adhesion formation by the use of polypeptides in the rat. Int J Colorectal Dis 2005; 21: 566-572.

12. Tingstedt B, Nehéz L, Lindman B, Andersson R: Efficacy of bioactive polypeptides on bleeding and intraabdominal adhesions. Eur Surg Res 2007; 39: 35-40.

13. Nehéz L, Vödrös D, Axelsson J, Tingstedt B, Lindman B, Andersson R: Prevention of postoperative peritoneal adhesions: Effects of lysozyme, poly-lysine and polyglutamate vs hyaluronic acid. . Scand J Gastroenterol 2005; 40: 1118-1123.

14. Fischer D, Li Y, Ahlemeyer B, Krigelstein J, Kissel T: In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterials 2003; 24: 1121-1131.

15. Hunter A: Molecular hurdles in polyfectin design and mechanistic background to polycation induced cytotoxicity. Adv Drug Deliv Rev 2006; 58: 1523-1531.

16. Morgan D, Larvin V, Pearson J: Biochemical characterisation of polycation-induced cytotoxicity to human vascular endothelial cells. J Cell Sci 1989; 94: 553-559.

17. Jorquera RA, Berrios J, Reyes JG: Permeability changes induced by polylysines in rat spermatids. Biol Cell 2002; 94: 223-41.

18. Salvi M, Toninello A: Effects of polyamines on mitochondrial Ca2+ transport. Biochimica et Biophysica Acta (BBA) - Biomembranes 2004; 1661: 113-124.

19. Lv H, Zhang S, Zhang S, Wang B, Cui S, Yan J: Toxicity of cationic lipids and cationic polymers in gene delivery. J Control Release 2006; 114: 100-109.

20. Moreau E, Domurado M, Chapon P, Vert M, Domurado D: Biocompatibility of polycations: In vitro agglutination and lysis of red blood cells and in vivo toxicity. J Drug Target 2002; 10: 161-173.

21. Holmdahl L, M MA-J, Risberg B: Experimental models for quantitative studies on adhesion formation in rats and rabbits. Eur Surg Res 1994; 26: 248-256.

22. King A, Strand B, Sandler S: Improvement of the biocompatibility of alginate/poly-L-lysyne/alginate microcapsules by the use of epimerized alginate as coating. J Biomed Mater Res 2003; 64A: 533-539.

23. Takei Y, Maruyama A, Ferdous A, Nishimura Y, Kawano S, Ikeijima K, Okomura S, Asayama S, Nogawa M, Hashimoto M, Makino Y, Kinoshita M, Watanabe S, Akaike T, Lemasters JJ, Sato N: Targeted gene delivery to sinusoidal endothelial cells: DNA nanoassociate bearing hyaluran-glycocalyx. FASEB J 2004; 18: 699-701.

24. Georgnaras I, Yoon Y, Belk K, Smith G, Sofos J: Antimicrobial activity of epsilon-polylysine against Escherichia Coli O157:H7, Salmonella Typhyrium and Listeria monocytogenes in various food extracts. J Food Sci 2007; 72: 330-4.

25. Billinger M, Buddeberg F, Hubbell J, Elbert D, Schaffner T, Mettler D, Windecker S, Meier B, Hess O: Polymer stent coating for prevention of neointimal hyperplasia. J Invasive Cardiol 2006; 18: 423-426.

26. Li C, Yu D, Newman A, Cabral F, Stephens C, Hunter N, Milas L, Wallace S: Complete regression of well-established tumors using a novel water-soluble poly(l-glutamitic acid)-paclitaxel conjugate. Cancer Res 1998; 58: 2404-9.

27. Moroson H: Polycation-treated tumor cells *in vivo* and *in vitro*. Cancer Res 1971; 31: 373-380.

28. Shih I, Van Y, Shen M: Biomedical applications of chemically and microbiologically synthesized poly(glutamatic acid) and poly(lysine). Mini Rev Med Chem 2004; 4: 179-188.

29. Sela M, Katchalski E: Biological properties of poly-a-amino acids on molecular size. Adv Protein Chem 1959; 14: 391-478.

30. Kenausis G, Voros J, Elbert D, Huang N, Hofer R, Ruiz-Taylor L, Textor M, Hubbell J, Spencer N: Poly)l-lysine)-g-Poly(ethylene glycol) Layers on metal oxide surfaces: Attachement mechanism and effects of polymer architecture on resistance to protein adsorption. J Phys. Chem B 2000; 104: 3298-3309.

31. Strand B, Ryan L, Veld PI, Kulseng B, Rokstad A, Skjåk-Brek G, Espevik T: Poly-L-lysine induces fibrosis on alginate microcapsules via the induction of cytokines. Cell Transplant. 2001; 10: 263-275.

32. Menger FM: Migration of poly-l-lysine through a lipid bilayer. J Am Chem Soc 2003; 125: 2846-2857.

33. Hong S, Leroueil P, Janus E, Peters J, Kober M, Islam M, Orr B, Jr JB, Holl MB: Interaction of polycationic polymers with supported lipid bilayers and cells: nanoscale hole formation and enhanced membrane permeability. Bioconjugate Chem 2006; 17: 728-734.

34. Vries AD, Feldman J, Stein O, Stein Y, Katchalski E: Effects of intravenously administered poly-D-L-lysine in rats. Proc Soc Exp Biol Med 1953; 82: 243-247.

35. Moreau E, Ferrari I, Drochon A, Chapon P, Vert M, Domurado D: Interactions between red blood cells and a lethal partly quaternized tertiary polyamine. J Control Release 2000; 64: 115-128.

36. Andrews DW, Ottensmeyer FP: Electron microscopy of the poly-L-lysine alpha-helix. Ultramicroscopy 1982; 9: 337-48.

37. Chittchang M, Alur HH, Mitra AK, Johnston TP: Poly (L-lysine) as a model drug macromolecule with which to investigate secondary structure and membrane transport, part 1: Physicochemical and stability studies. J Pharm Pharmacol 2002; 54: 315-23.