Biodegradable Graft Polyesters Based on Copolymer of Lactic Acid and Glycolic Acids Grafted onto Poly(vinyl alcohol) for Oral Vaccine Delivery

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SUMMARY: Small nanospheres prepared by spontaneous polymer - protein self-assembling are an attractive concept for the preparation of nanoscale drug delivery systems, since the use of solvents and surfactants can be avoided. For this purpose, polyesters were prepared by grafting poly(lactic acid-*co*-glycolic acid) (PLGA) chains onto poly(vinyl alcohol) (PVAL) or the negatively charged sulfobutylated poly(vinyl alcohol), P(SBVE). Adjustment of PLGA chain lengths by feed composition allowed to modify polymer properties, such as molecular weight and solubility. While polyesters with a chain length of 5-10 lactic or glycolic acid units showed on average good solubility in acetone, further chain length reduction yielded water-soluble polymers. In aqueous solution, a lower critical solution temperature was observed.
Spontaneous formation of colloidal polymer - protein conjugates with a variety of proteins, such as tetanus toxoid, recombinant human nerve growth factor and insulin was investigated. Sizes ranging from ca. 100 nm to several µm and protein loading of up to 200 % could be attained by changing factors, such as pH, temperature and polymer type. Complex formation was fully reversible. Bioadhesion in a Caco-2 cell culture model and measurable antibody titers in mice using tetanus toxoid - polymer conjugates suggest that these polymers could be of interest for protein delivery and mucosal vaccination.

INTRODUCTION

Recombinant DNA technology provides a wide variety of proteins, peptides and oligonucleotides, which could be of considerable therapeutic interest. Due to their size and lability, they require suitable delivery systems to exploit their potential. The protection of biologically active proteins against denaturation and enzymatic degradation is an important issue. Possible strategies based on polymeric carriers for oral and parenteral delivery of proteins include: (a) modification of biologically active compounds with polymers, (b) encapsulation of hydrophilic macromolecules into micro- or nanospheres and (c) adsorptive

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drug loading onto the surface of nanospheres. Covalent modification of proteins with polymers, e.g. by PEGylation, can be used for altering and controlling their pharmacokinetics, biodistribution and often toxicity [1]. Polymer - drug conjugates have shown persistently high plasma levels after parenteral application, even gradually accumulating in peripheral tumors, whereas the cationic ones are trapped by the liver and blood vessels and rapidly cleared from circulation [2]. There are two limitations of the polymer conjugation strategy: For one, the protein must contain suitable functional groups for derivatization. Second, modification of those groups can affect the biological activity in a negative sense.

Using micro- and nanoencapsulation, depot systems can be prepared, but proteins are also very susceptible to harsh conditions during preparation, e.g. organic solvents and shear forces in the emulsification processes [3]. Moreover, biocompatibility and biodegradability, balance of hydrophilic and hydrophobic moieties, etc., leading to insufficient drug release can be an issue. Especially in peroral drug delivery, very small lipophilic polystyrene nanospheres seem to allow mucosal particle absorption [4]. Negatively charged nanospheres prepared from sebacic and fumaric acid copolymers [5] and anionic liposomes [6] showed intestinal uptake.

A promising strategy could be based on polymeric carriers combining all the properties described above, namely biodegradability and the possibility of engineering the nanosphere surface by manipulation of the balance of hydrophilic and hydrophobic domains as well as by surface charges. This technological platform would avoid organic solvents, emulsification or shear forces and, therefore, would rely on self-assembly of a protein and a carrier system in an aqueous environment. Such macromolecular self-assembling systems have recently attracted increasing attention as carriers for drug delivery [7,8].

We have investigated nanospheres with designed surface properties prepared from biodegradable comb polyesters, consisting of poly(lactide-*co*-glycolide) brush grafted onto charged or uncharged poly(vinyl alcohol) backbones envisaged for peroral and nasal vaccination [9,10]. Here we report the preparation of water-soluble comb polyesters [11] and their potential use for protein delivery systems.

MATERIALS AND METHODS

Starting materials

Poly(vinyl alcohol) (PVAL, M_w 15 000, degree of hydrolysis 88 %) was obtained from Fluka and thoroughly dried at 80 °C in vacuo. DL-lactide and glycolide (Boehringer Ingelheim, S-grade) were recrystallized twice from dry ethyl acetate. All other materials were of analytical grade and were used as received.

Polyelectrolyte backbones

Poly(4-sulfobutyl vinyl ether), P(SBVE), was prepared from PVAL under anhydrous conditions in dry nitrogen atmosphere, as reported recently [10,11]. Briefly, PVAL was activated with dimsyl sodium, obtained by the reaction of DMSO with sodium hydride, and etherified with butane-1,4-sultone at room temperature. Ultrafiltration was performed four times with each sample (initial concentration: 200 mg polymer in 10 ml water) with an Amicon ultrafiltration cell 8010 equipped with a YM1 filter membrane (Amicon, cut-off 1000).

Polyesters

Biodegradable comb polyesters were prepared by grafting poly(lactic acid-*co*-glycolic acid) onto the PVAL or P(SBVE) backbone [10,11]. Briefly, ring-opening polymerization of the lactones, L-lactide or DL-lactide and glycolide, in the presence of different core polymers with stannous octoate as catalyst was performed in the bulk. Reaction time was 10 min at 170 °C to achieve sufficient solubility of the polymers in the melt of the lactones, then the reaction was allowed to continue for additional 3 h at 150 °C. Purification of the water-soluble polyesters was performed by ultrafiltration.

Polymer characterization

Size exclusion chromatography (SEC) and static light scattering (SLS): 0.5% (w/v) polymer solutions were injected into a thermostatted (35 °C) Merck-Hitachi system with a differential refractometer (RI 71) and a MiniDawn light scattering detector (Wyatt Technology Corporation) (100 µl K5 cell, laser wavelength 690 nm, laser power 30 mW, detecting angles 45°, 90° and 135°). Chromatograms were obtained with degassed eluents at a flow rate of 1 ml/min. For dichloromethane and acetone, a Lichrogel PS mix and a PS 40 (10 µm) Merck column; for aqueous SEC analysis, a column combination Suprema 10µ and Suprema linear 10µ - 8x300 mm (PSS) were used.

NMR spectroscopy was performed at 35 °C with 6% (w/v) polymer solutions in fully deuterated solvents (acetone-d_6, $CDCl_3$, DMSO-d_6, D_2O). 400 MHz ^1H and 100 MHz ^{13}C NMR spectra were recorded with a Jeol GX400 Delta N FT spectrometer, 500 MHz ^1H and 125 MHz ^{13}C NMR spectra with a Jeol LA500 eclipse - Delta FT spectrometer.

Intrinsic viscosities were determined using an Ubbelohde viscosimeter (Schott Geräte, Germany) with aqueous 0.5 M $NaNO_3$ solutions at 25 °C at four concentrations at least.

Turbidity measurements as a function of temperature for LCST determination were performed at different wavelengths with a Shimadzu UV-VIS spectrophotometer UV-160 or with a Zetasizer 4 (AZ110 cell, Malvern Instruments, 630 nm, 90°).

Polymer - protein complexes

For preparation of complexes, 100 mg of polymer was dissolved in 1 ml of an isotonic (0.15 M) phosphate buffer saline solution (PBS) of defined pH. In a typical example, 100 µl of this polymer stock solution was transferred into a 1500 ml Eppendorf vial and diluted with 800 µl of PBS. Finally, 100 µl of a 10 mg/ml protein stock solution in PBS was added and vortexed for 10 s. For uncharged polymers, temperature was then kept at 37 °C for 1 h. Each experiment was performed in triplicate.

Drug loading and release (at 37 °C) were determined in the supernatant, after centrifugation of the complexes, photometrically at 280 nm (tetanus toxoid, Ttx) and 491 nm (fluorescein isothiocyanate (FITC-BSA) with a Shimadzu UV-VIS spectrophotometer UV-160 in triplicate.

Colloid sizes and distributions were measured in triplicate at 25 °C in aqueous dispersions (200 µg/ml) by photon correlation spectroscopy with a Zetasizer 4 (AZ110 cell, Malvern Instruments, 90°, sample time 120 µs, serial mode, 4 mW laser, 64-channel correlator, multimodal analysis).

Isothermal titration calorimetry was performed with an MCS-ITC equipment (Microcal Inc.; 25 °C, cell volume 1351.3 µl, stirring syringe 250 µl, 400 rpm, 10-µl injections every 250 s) and data processing with software Origin 3.5 (Microcal). Polymer solutions of known concentrations were titrated at different solution pH with protein solutions of known concentrations. All experiments were corrected using measured values of dilution enthalpy.

Non-reducing SDS PAGE and Native PAGE experiments were carried out with a Phast-System (separation and development unit, LKB Pharmacia). Protein separation and gel development were performed using the Pharmacia methods ‘separation’ and ‘development technique files’. For protein separation, 1 µl of the sample was applied onto foil-supported poly(acrylamide) gels (Phast-Gel gradient 8-25, SDS-PAGE: Phast-Gel SDS buffer strips, Native PAGE: Phast-Gel Native buffer strips, Pharmacia). For calibration, a high- and low-molecular-weight kit for electrophoresis (Pharmacia) were used. After electrophoresis, staining was performed with Coomassie Blue (PhastBlue R, Pharmacia), the residual dye was removed by washing with a mixture of methanol, acetic acid and water (3:1:6) and the gels were fixated.

For statistical design and analysis (factorial screening design with 3 center points), the software Statgraphics Plus for Windows 2.1 (Statistical Graphics Corp., Rockville, USA) was used.

Animal studies

Female Balb/c mice, 7-9 weeks of age, weighing 16-22 g, were obtained from Harlan-Winkelmann (Germany). Three groups of mice were used in these experiments. Complex dispersions were compared with conventional alum-adsorbed and free Ttx. Mice were randomized, pooled into groups of 10 animals and immunized in three consecutive weeks (day 1, 8, 15) by peroral (p.o.) application of 200 µl of 5LF Ttx containing colloidal polyelectrolyte complexes. The i.p. inoculations with 200 µl of Tetanol® were performed as a positive control. Animals were bled in week 0 and 4. All sera were assayed in duplicate for Ttx-specific IgG and IgA antibody responses using an ELISA technique.

RESULTS AND DISCUSSION

The branched polyesters (Table 1), characterized by a three-dimensional architecture, were obtained by grafting of short poly(lactic acid-*co*-glycolic acid) (PLGA) chains onto hydrophilic polymer backbones. They were prepared by melt polymerization of the lactones, lactide and glycolide, in the presence of different polyols [12]. The backbone polyols used were an unmodified poly(vinyl alcohol) (PVAL) of M_w 15 000 and PVAL bearing negative charges. The charged groups were introduced by reacting the PVAL with butane-1,4-sultone.

We have investigated structure modification of PLGA [9-16], since these polymers offer additional options to manipulate their balance of hydrophobic and hydrophilic domains. In particular, variation of the PLGA side chain lengths grafted onto the backbone of polyols allows to adjust properties, such as molecular weight (M_w), degree of crystallinity, glass transition temperature (T_g) and solubility [12]. As outlined in Table 1, the best solvent for polymers with PLGA chain lengths in the range of ca. 10 to 30 repeating units was found to be dichloromethane. The lipophilic polyesters have demonstrated their potential in drug delivery of protein-loaded microspheres [14-16]. A reduction in the side-chain lengths to average 5-10 units increased polymer amphiphilicity and resulted in acetone solubility, which made these polymers ideal candidates for nanosphere preparation [10,13]. Polymers with even shorter PLGA chains (ca. 3-5 units) became water-soluble [11].

Figure 1a demonstrates, that a reduction in the side chain lengths causes a decrease in the intensity of the PLGA-chain ^{1}H NMR signals (ppm: 1.45, CH_3, lactic acid; 4.8, CH_2, glycolic acid; 5.16, CH, lactic acid) and an increase in the terminal OH groups of PLGA (4.2, **CH_2**OH; 4.35, **CH**(CH_3)OH; 1.28, CH(**CH_3**)OH). The PVAL signals were (ppm): 1.9, **CH_3**COO; 1.7, **CH**-OCO; 1.3-1.5, CH_2. By comparison of the signal intensities of the PLGA chain and its end groups, average PLGA chain lengths could be calculated. Some selected polymers were

Table 1: Physico-chemical properties of graft polyesters

No.	Polymer	Degree of PVAL sulfobutylation [a] (%)	PVAL M_w [a] x 10^{-3}	PLGA chain M_n [b]	LA or GA units per chain [b]	Polymer M_n [b] x 10^{-3}	LA:GA units [b] (mol%)	Best solvent
1	PVA-*g*-PLGA	-	15	4000	32	1250	51 : 49	CH_2Cl_2
2	PVA-*g*-PLGA	-	15	1100	9	360	51 : 49	CH_2Cl_2 : acetone 1:1
3	PVA-*g*-PLGA	-	15	590	5	238	50 : 50	acetone
4	PVA-*g*-PLGA	-	15	390	3	134	50 : 50	acetone
5	PVA-*g*-PLGA	-	15	50[c]	0.8[c]	30[c]	50 : 50[c]	water
6	PVA-*g*-PLGA	-	15	37[c]	0.4[c]	26[c]	50 : 50[c]	water
7	PVA-*g*-PLGA	-	15	30[c]	0.3[c]	24[c]	50 : 50[c]	water
8	PVA-*g*-PLGA	-	15	21[c]	0.2[c]	21[c]	50 : 50[c]	water
9	P(SBVE)-*g*-PLGA	14	19.9	590	5	172	53 : 47	acetone
10	P(SBVE)-*g*-PLGA	14	19.9	50[c]	0.4[c]	33[c]	50 : 50[c]	water
11	P(SBVE)-*g*-PLGA	27	26	840	7	210	52 : 48	acetone
12	P(SBVE)-*g*-PLGA	27	26	120[c]	2[c]	52[c]	50 : 50[c]	water : acetone
13	P(SBVE)-*g*-PLGA	27	26	60[c]	0.5[c]	35[c]	50 : 50[c]	water
14	P(SBVE)-*g*-PLGA	43	33.6	1100	9	221	53 : 47	acetone
15	P(SBVE)-*g*-PLGA	43	33.6	80[c]	0.6[c]	35[c]	50 : 50[c]	water

[a] From elemental analysis; [b] from NMR analysis; [c] calculated from the monomer feed since NMR signals were too broad

analyzed by a combination of SEC and static light scattering (SLS) to characterize their molecular weights. The M_ws attained values up to several hundred thousands [12] confirming that the M_ws directly followed the feed compositions. As outlined in Fig. 1b, lower M_ws are obtained with higher polyol backbone contents in the feed.

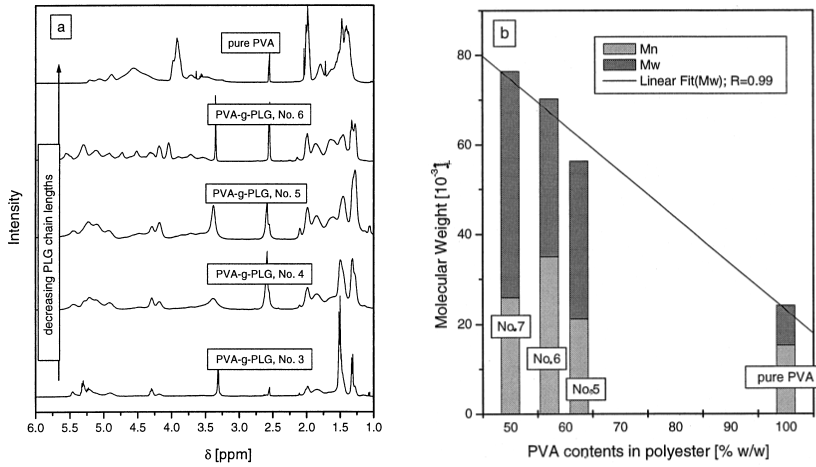

Figure 1: Characterization of branched polyesters by (a) ^{1}H NMR, (b) SEC-SLS.

All water-soluble polyesters exhibited a low critical solution temperature (LCST) and precipitation at higher temperatures, already known for PVAL. As outlined in Fig. 2, LCST was a linear function of the PLGA chain length. The shorter the side chains, the more hydroxy groups are in the polymers and, therefore, the better is their solubility, causing higher precipitation temperatures. At high polymer concentrations, temperature-induced precipitation was visible even for polymers with anionic backbone, although they generally exhibited better solubility owing to the charged groups that raised LCST to higher values. Nevertheless, these temperatures were still in a quite attractive range near physiological conditions. The existence of an LCST offered the possibility of preparing a hydrogel type of protein delivery system with these polyesters. Combining a protein and a polymer in solution, then raising temperature above the LCST (e.g. by parenteral application) resulted in precipitation of the polymer partly including protein molecules.

The uncharged polymers gave larger inclusion-like aggregates with, e.g., fluorescently labeled bovine serum albumin (FITC-BSA) on a temperature switch. The situation changed when a negatively charged polymer and protein were combined in solution: temperature- independent spontaneous self-assembly of very small complexes occurred. Various high-resolution

microscopic techniques, such as field-emission scanning electron microscopy and freeze-fracture transmission electron microscopy, revealed larger (µm range) precipitates with the uncharged polymers while very small colloids, only a few hundreds of nanometres in size, were found with anionic polymers (data not shown).

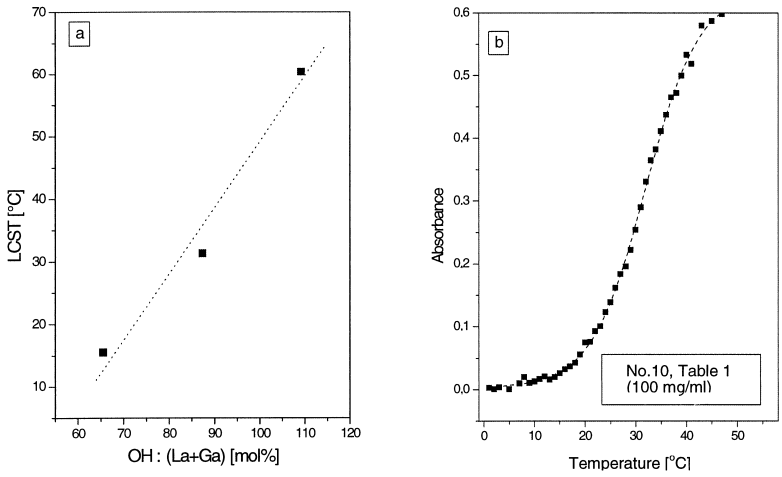

Figure 2 : (a) Lower critical solution temperature of P(SBVE)-*g*-PLGA as a function of PLGA chain length and (b) turbidity of the polymer solution as a function of temperature.

The complex composition was investigated after purification and isolation by several centrifugation and washing steps. The presence of the polymer could be easily detected by ^{1}H NMR after redissolving the complex in deuterated DMSO. The presence of the protein, in this case iron-containing cytochrome C (CytC), was confirmed using an electron microscope equipped with energy-dispersive x-ray microanalysis (EDX) allowing the detection of the protein iron atom in the complexes at ca. 6.25 - 6.5 keV (data not shown).

A factorial design is showing this expected influence of solution pH and ionic strength (amount of additional NaCl) on the complex formation of BSA with an anionic polymer at a constant polymer-to-protein ratio. The DLS intensity, as an indirect measure of the number (and size) of the formed polyelectrolyte complexes between polymer and protein, decreased with increasing pH value. The strongest reaction was seen at the lowest pH investigated (pH 3), where the polymer is negatively and the protein positively charged. The intensity significantly decreased at a pH near the isoelectric point of the protein, where its overall charge is zero, further decreasing with increasing solution pH. At pH ca. 6-7, no reaction was observable. These results demonstrate one of the major advantages of the polymer if applied

parenterally: the negative charge of the polymer and moderate pH under physiological conditions will prevent complexation of serum albumin which itself is anionic. The drug loading was a linear function of solution pH, while no influence of its ionic strength was observed. The highest loadings were achieved under acidic conditions; the loadings linearly decreased with increasing pH, reflecting the results on complex formation and sizes. Investigation of the complex stability by supplementary addition of sodium chloride to increase the solution ionic strengths after complex formation revealed no major changes. Neither colloid sizes nor DLS intensity were influenced, except for the dilution effect, indicating strong binding between the complex partners. Control of the complex size and concentration as well as protein loading by factors, such as pH and ionic strength of the solution, could be another advantage of controlled drug delivery. It is well known that small particles are most effective in, e.g., mucosal (oral, nasal) application.

To further investigate the nature of the reaction between the polymers and proteins, isothermal microtitration calorimetry was performed. Polymer solutions of known concentrations and pH were degassed and titrated with proteins under stirring at constant temperature. Titration of the uncharged polymers with proteins revealed no major heat changes except dilution effects, indicating that, at constant temperature below LCST, no reaction occurred. With the negatively charged polymers, strong heat changes during titration were observed due to the complexation. The heat changes associated with the complexation were endothermic, decreasing monotonically upon successive protein injections. These results are in agreement with literature data, e.g., adsorption of albumin onto negatively charged surfaces [17,18]. The complexation is driven by a large increase in entropy.

From a series of experiments with different proteins, a dependence of the complex composition and the size/molecular weight of the protein was found (Fig. 3), already postulated from the drug loading results described above. It is worth noting that the mean values and standard deviations in this plot are not derived by repeating a single experiment several times, but by a series of experiments using different concentrations of the two complex constituents. With CytC of molecular weight 12 000, two protein molecules seem to share one polymer molecule. A 1:1 reaction was found for BSA and a 2 : 1 reaction for Ttx with an approximate M_w of ca. 150 000. In other words, a maximum drug loading of 50 wt% for Ttx could be achieved for BSA, 100 wt% and even 200 wt% for CytC, demonstrating the potential of this type of drug delivery system, compared with micro- and nanospheres, which normally allow drug loadings 1 – 10 %.

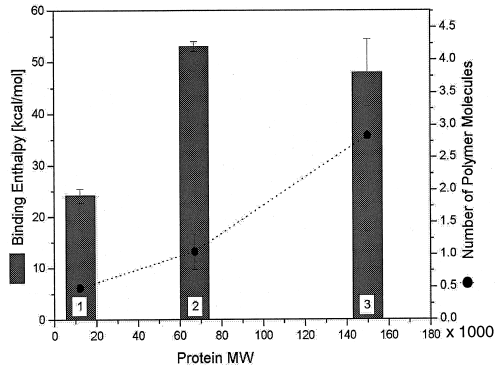

Figure 3 : Isothermal microtitration calorimetry of P(SBVE)-*g*-PLGA (No.10, Table 1) with different proteins: *1* cytochrome c, *2* bovine serum albumin and *3* tetanus toxoid.

To investigate the influence of pH on the complex stability and protein release, a series of FITC-BSA complexes was prepared at pH 3 and purified by three centrifugation/washing cycles. The purified samples were then immersed in buffer solutions of different pH (3, 6, 7.4) at 37 °C. At pH 3, negligible protein release was observed. As outlined in Fig. 4a, the complexes once prepared remain stable at least for several days. Raising pH to 6 resulted in a slow but continuous release of the complexed protein. A further increase in pH to physiological conditions (PBS 7.4) caused a fast and nearly linear release of the whole protein amount in less than 24 h.

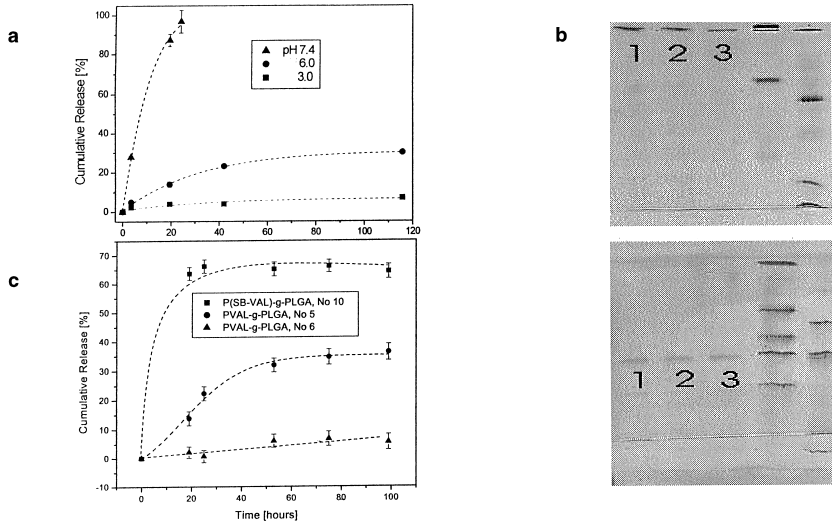

Figure 4 : In vitro release profiles of (a) bovine serum albumin from P(SBVE)-*g*-PLGA complexes as a function of pH, (b) stability of BSA after desorption at different pH and (c) release of tetanus toxoid as a function of different polymer compositions.

The integrity of BSA was investigated by native and SDS poly(acrylamide) gel electrophoresis (PAGE). Compared with the original protein, they were in a non-aggregated, native form as demonstrated in Fig. 4b. The complex formation was fully reversible, the presence of the polymer seems to have inhibited BSA self-aggregation and denaturation even under acidic conditions and at elevated temperatures.

Similar results were obtained with tetanus toxoid containing colloids (Fig. 4c): no release at acidic pH, significantly accelerated at pH values above the isoelectric point of the protein. The release from the inclusion-like complexes with uncharged polymers was found to be pH-independent and much slower. It seemed to be mainly an adsorption/desorption equilibrium, controlled by the polymer composition. Higher proportions of the hydrophilic backbone in the polymer (shorter PLGA chains) enabled slightly faster release through porous hydrated PVAL domains, while in the case of higher-molecular-weight polymers, release rates were lower.

Peroral absorptions of the complexes were investigated in mice using tetanus toxoid (Ttx) as a model antigen. Three doses of the complexes containing 5LF Ttx were orally administered in the course of three weeks (n=10). The stomach pH was not buffered in the in vivo experiments. As can be seen in Fig. 5, an increase in serum IgA and IgG titers could be achieved after peroral administration.

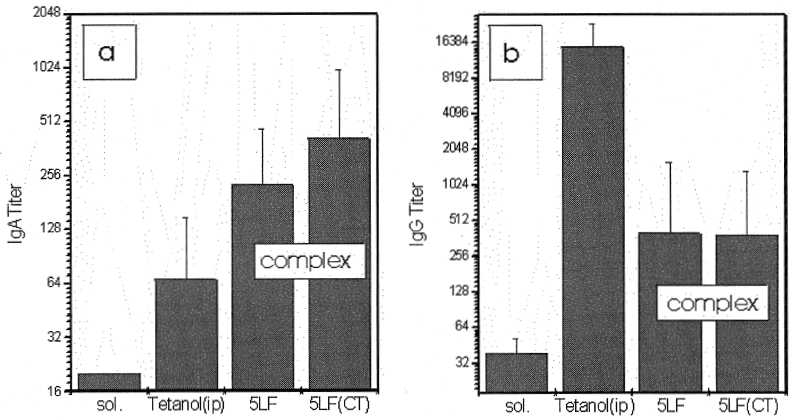

Figure 5 : Immune response to P(SBVE)-*g*-PLGA complexes with Ttx in mice after peroral administration (n=10) compared with intraperitoneal injection and solution of Ttx. The IgG titers (a) and IgA titers (b) were measured in serum.

While no substantial titers were observable after oral administration of solution, the well-documented increase in IgG after intraperitoneal injection of alum-adsorbed tetanus toxoid (Tetanol) served as control. An about 4 to 6 times higher IgA titer was achieved with the

colloids compared with Tetanol, suggesting potential activity in mucosal vaccination. While these preliminary results need to be confirmed by further experiments, they demonstrate the potential of colloidal carriers for mucosal vaccination using biodegradable branched polyesters as carrier systems.

CONCLUSIONS

A novel class of water-soluble graft copolymers containing poly(lactic acid-*co*-glycolic acid) of different lengths, grafted onto a poly(vinyl alcohol)-based backbone was obtained. The maximum graft lengths for water solubility should not exceed five lactic or glycolic acid units per chain. The polymers showed stimuli-sensitive properties allowing the formulation of protein drug delivery systems triggered by temperature or electrostatic interaction.

Spontaneous and reversible formation of complexes with several proteins was observed. In the case of polyelectrolyte complexes with charged polymers, a suitable candidate could be any protein if processed at a pH below its isoelectric point. In particular basic proteins, such as cytochrome C, allow polyelectrolyte complexation at pH values near physiological conditions.

While the colloid sizes could be adjusted in the range from ca. 100 nm to several µm by concentration, solution pH and ionic strength, the release rates of the complexed proteins were determined by pH. Data from a peroral vaccination experiment in mice suggest that these polymer - protein complexes merit further investigation as mucosal vaccine delivery systems.

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