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Lactic acid triggers, in vitro, thiomersal to degrade protein in the presence of PLGA microspheres

Jocimara A.M. Namur [a], Célia S. Takata [b], Ana M. Moro [c], Mário J. Politi [d], P. Soares de Araujo [d], Iolanda M. Cuccovia [d], M.H. Bueno da Costa [a,*]

[a] *Lab. de Microesferas e Lipossomos, C. de Biotecnologia, São Paulo, SP, Brazil*
[b] *Divisão de Desenvolvimento Tecnológico e Produção, São Paulo, SP, Brazil*
[c] *Lab. de Biofármacos em célula animal, I. Butantan, Av. Vital Brasil 1500, 05503-900 Butantan, São Paulo, SP, Brazil*
[d] *I. de Química, USP, Av. Lineu Prestes 748, São Paulo, SP, Brazil*

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Abstract

Microspheres of polymers like poly(lactic-co-glycolic acid) (PLGA) have been studied as a vehicle for controlled release vaccines. They require materials and processes that might change the protein antigenicity. Lactic acid is produced during microsphere degradation that occurs in tandem with protein liberation. In addition, most of the proteins that have been used in microencapsulation studies contain Thimerosal®(TM) and this can introduce another undesirable effect for their stability. We demonstrated in vitro that the thiosalycilic acid (TSA), produced after the reduction of TM by lactic acid, reduces the S–S bridge of the previously incubated diphtheric toxoid (Dtxd). This reduction is immediately followed by blocking the two –SH formed by the same TSA molecules. In the light of these conclusions it is necessary now, to reinterpret the in vitro protein degradation-stabilization data in the presence of PLGA microspheres, mainly for those proteins which contain S–S. We propose that all the PLGA microspheres microencapsulation studies and protein structural considerations should be done in the absence of TM as preservative.

Keywords: PLGA/protein interaction; PLGA vaccine stability; Thiomersal and microencapsulation; Protein instability; Protein degradation

1. Introduction

Microspheres of biodegradable polymers like poly(lactic-co-glycolic acid) (PLGA) have been studied as a vehicle for single-dose vaccines. They require materials and processes that might reduce or destroy the antigenicity of the encapsulated antigen (Johansen et al., 1998; Zhu and Schwendeman, 2000; Kang and Schwendeman, 2002) and consequently very few peptides and proteins have been successfully delivered using this polymer (Furr and Utchinson, 1992; Johnson et al., 1996; Zhu et al., 2000). Besides the intrinsic instability of proteins, moisture induced aggregation and/or degradation, acidic microenvironment due to polyester degradation, and polymer/protein interactions have all been considered as major factors contributing to the incomplete protein release from PLGA microspheres (Kang and Schwendeman,

* Corresponding author. Tel.: +55-11-3726-7222x2265; fax: +55-11-3726-1505.
E-mail address: bdacosta@edu.usp.br (M.H.B. da Costa).

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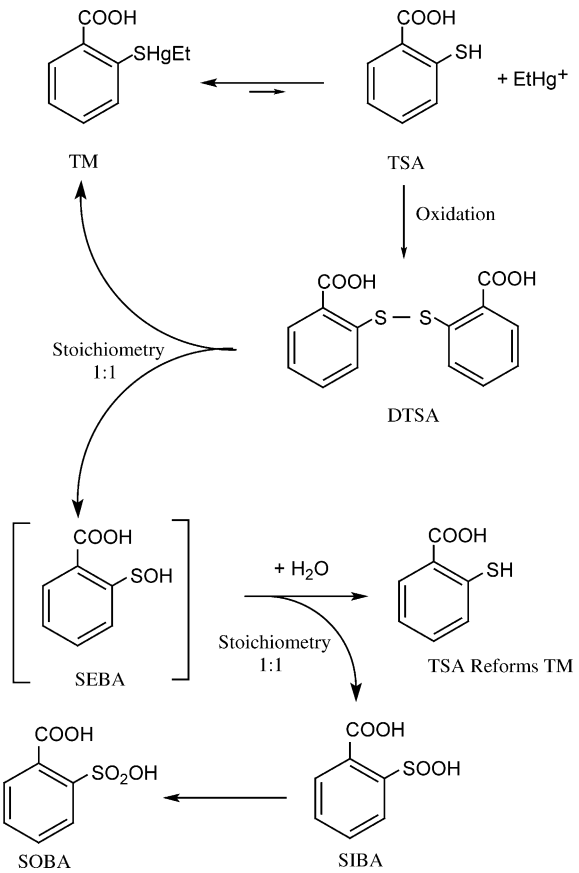
2002). In addition most of the antigenic proteins that have been used in microencapsulation studies in vitro contain Thimerosal® as preservative and this can introduce another undesirable effect for their stability as will be demonstrate here.

Thimerosal® (TM)—also known as thiomersal, Merthiolate® or sodium ethylmercuri-thiosalicylate—is a water-soluble derivative complex of thiosalicylic acid (TSA) that has been used as bactericide in parenteral vaccines and ophthalmic products for decades. It has been reported that this preservative can be decomposed by oxidation to 2,2′-dithiosalicylic acid, ethyl mercuric ion, 2-sulfenobenzoic acid, 2-sulfobenzoic acid and 2-sulfinobenzoic acid (Tleugabulova and Pérez, 1996; Tan and Parkin, 2000) (Scheme 1). The complex chemistry of degradation in aqueous media of Thimerosal® has not been thoroughly studied in vaccines (Tleugabulova and Pérez, 1996). The importance of the problem is related to the formation of unknown compounds at advanced stages of degradation, which could produce adverse reactions in humans exposed to a drug containing the degraded TM (Tan and Parkin, 2000).



Scheme 1. Proposed route for Timerosal® degradation (adapted from Tleugabulova and Pérez, 1996; Tan and Parkin, 2000). The possible compounds originated from TM degradation are TSA, ethyl mercuric ion (EtHg), 2,2′-dithiosalicylic acid (DTSA), 2-sulfenobenzoic acid (SEBA), 2-sulfinobenzoic acid (SIBA) and 2-sulfobenzoic acid (SOBA).

The Thimerosal® degradation is strongly influenced in a complex manner by the presence of other species in the media, as examples, chloride promotes degradation and EDTA stabilization (Tan and Parkin, 2000). TM is a versatile sulphydryl reagent, calcium mobilizer and cell function-modulating agent (Philippe, 1995; Elferink, 1999; Dogar et al., 2002). The ability of TM to act as sulfhydryl group is related in the literature to the presence of Hg (Elferink, 1999; Dogar et al., 2002). Here we will study, in vitro, the interaction of diphtheria toxoid (Dtxd), an S–S containing protein (Rappuoli, 1997) with TM and PLGA microspheres. We propose that, in vitro, thiosalycilic acid, produced after the reduction of Thimerosal® by the lactic acid, reduces the S–S bridges of the diphtheria toxoid, producing Fragment A (FA) and Fragment B (FB).

2. Materials and methods

2.1. Materials

Diphtheria toxoid was produced and purified by Instituto Butantan. PLGA 50:50 (poly(lactide-co-glycolide), LactelBP 0100®, M_w 45,000–75,000), Lactic acid reagent (a solution of lactic acid oxidase, peroxidase, chromogen precursors and buffer, pH 7.2) and lactic acid standard solution, Thimerosal®, salicylic acid, 2-5-dithiobis-nitrobenzoic acid (DTNB) were purchased from Sigma, poly(vinylalcohol) (PVA), M_w 49,000 was from Aldrich Chemical Company (USA), membranes 0.22 μm were from Millipore. A Shimadzu HPLC, Model LC-10VP equipped with UV Detector, Model SCL-10AVP, a QC-PAK, GFC 300 column (15 cm × 7.8 mm) were purchased from Shimadzu Co., Japan. All other reagents were of analytical grade.

2.2. Methods

2.2.1. PLGA microspheres preparation

The PLGA particles were prepared using the water in oil/in water ($(W_1/O)/W_2$) double emulsification solvent evaporation method (Costa and Fattal, 2000). Briefly, under strong agitation, 125 μl of PBS containing 37 μM Thimerosal® were added to 200 mg of PLGA previously dissolved in 2 ml of CH_2CL_2. This mixture was then emulsified at 24,000 rpm for 2 min in an Ultraturax® emulsifier. The emulsion was quickly added to 40 ml of 0.5% PVA and submitted to emulsification at 19,000 rpm for 2 min. The solvent was evaporated by gentle stirring for 3 h at 1000 rpm in a Heidolph® RZR 2051 helix evaporator. The microspheres were collected by centrifugation for 10 min at 2000 g, rinsed with water three times and then resuspended with 2 ml of 0.1% PVA, freeze–dried for 24 h and stored at −20 °C. Phase contrast microscopy was used to observe microspheres morphology.

2.2.2. Dtxd interaction with PLGA microspheres

A solution (8 ml) of 9.4 mM Dtxd in PBS and 37 μM Thimerosal® was added to 40 mg of empty PLGA microspheres. The emulsion was incubated, under gentle agitation, during 8 weeks at 37 °C. Samples of 1 ml were collected and frozen weekly and, after 8 weeks, they were centrifuged. The supernatants were analyzed by measuring pH, lactic acid, protein degradation (by HPLC) and ELISA.

2.2.3. ELISA

Hundred miocroliters of samples were added to the ELISA plates and, after 18 h at 37 °C, the plates were washed three times with PBS containing 0.05% Tween 20. In the subsequent step, the plates were blocked with 100 μl of 0.1% BSA and incubated at 37 °C during 60 min. In the next step, the plates were washed three times with PBS containing 0.05% Tween 20. The anti-Dtxd sera (10 IU developed in horses[1]) were added. In the next step, the plates were washed three times with PBS containing 0.05% Tween 20. The anti-horse peroxidase conjugate was added 60 min later. In the next step, the plates were washed three times with PBS containing 0.05% Tween 20, and, the substrate TMB was added. After 15 min at room temperature, the reaction was stopped with 50 μl of 1 M H_2SO_4. The absorbance was automatically read at 450 nm in an absorbance reader Titerteck Multiscan MCC/340. Antibody titers were expressed as $\log_2$ titer per microgram of protein, where titer is the reciprocal serum dilution factor giving an absorbance value of 20% of the saturation value.

2.2.4. Lactic acid assay

The sample supernatants (50 μl) were added to 50 μl of lactic acid reagent. The reaction mixture was incubated protected from light during 10 min at 37 °C. The absorbancies were automatically measured at 540 nm in an absorbance reader Titerteck Multiscan MCC/340.

2.2.5. Thimerosal® and Dtxd interaction in the presence of Lactic acid

Samples of 9.4 mM Dtxd were added to a solution of 3 mM Thimerosal®/10 mM lactic acid (final concentration of 3.7 mM Thimerosal®/10 μM lactic acid). Aliquots of 20 μl were applied automatically on a QC-PAK, GFC 300 HPLC gel filtration column (15 cm × 7.8 mm), with a flux of 0.6 ml/minute in a Shimadzu HPLC, Model LC-10VP equipped with UV Detector, Model SCL-10AVP. The protein and Thimerosal® concentrations were determined by measuring the absorbance at 269 nm. Briefly, UV spectra of samples of known protein concentrations (previously determined by the Lowry method) were determined in a Hitachi U-2000 Spectrophotometer. The Dtxd absorption peak was at 269 nm. The TM absorption spectrum is characterized by a large absorption band (220–300 nm), so it can be detected at this wavelength.

2.2.6. Thimerosal® and lactic acid interactions

Samples (250 μl) of 0.37 mM Thimerosal® were added to 750 μl of 1.0 M lactic acid (final concentrations 9.2 μM and 0.75 M, respectively). Immediately, aliquots of 20 μl were automatically applied on a QC-PAK, GFC 300 column at a flux of 0.6 ml/min. They were detected by the absorbance at 269 nm.

[1] The anti-Dtxd sera are normally produced and purified by the Divisão de Desenvolvimento Tecnológico e Produção of Instituto Butantan. Horses are immunized with Dtxd. After sera purification samples are analyzed by the Physico-chemical Quality Control section comparing with standard sera of known concentrations.

2.2.7. Fluorescence spectroscopy

The fluorescence emission spectra of 1.88 mM Dtxd samples in PBS containing 37 μM Thimerosal® were recorded with excitation at 269 nm and emission between 280 and 480 nm. The same spectra were determined in the presence of 0.9 M lactic acid.

3. Results

The interaction of Dtxd and PLGA microspheres was studied by incubating Dtxd with empty homogeneous particles of 12 μm diameter (Fig. 1).

The kinetics of lactic acid production and pH changes were followed by taking aliquots of the supernatants obtained after centrifugation of the reaction mixture. The pH decreased in concert with the lactic acid increase (Fig. 2).

Dtxd immunological activity, as measured by ELISA, decreased as function of time (Fig. 3).

Between the fifth and the eighth weeks, an increase in the amount of Fragment A and Fragment B was observed by HPLC (Fig. 4). The Dtxd dimer and monomer disappearance is concerted with the FB and FA increase.

The HCl that did not promote Dtxd degradation (Fig. 5) which occurred only in the presence of lactic acid and TM. Lactic acid addition to Dtxd accelerated the effect of Dtxd degradation (Fig. 5). In these conditions the degradation had a half-life of seconds. There is a marked change in the protein elution profile. Immediately after the addition of lactic acid, disappearance of TM, Dtxd dimer and Dtxd monomer (Fig. 5) was observed. The absorption spectrum for TM is characterized by a large absorption band (228–300 nm). Therefore, it can be measured at 269 nm, the wavelength used for Dtxd, in the HPLC experiments. Since it is not possible to do a mass balance between the reagents and the

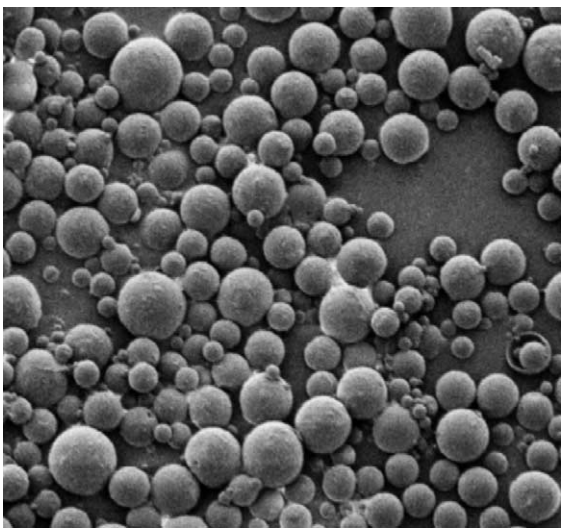


Fig. 1. Scanning electronic microscopy of a typical empty PLGA microsphere preparation.

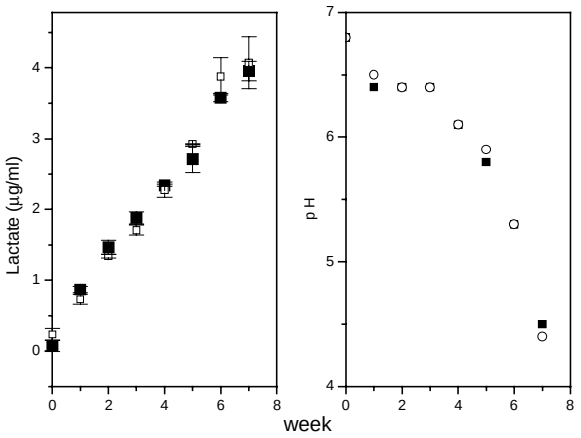


Fig. 2. Kinetics of lactate liberation and pH variation. The lactate and pH were measured in the presence or absence of Dtxd. Blank microspheres (■) and microspheres in the presence of Dtxd (○).

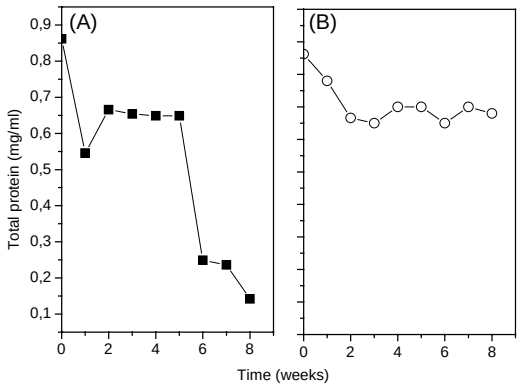


Fig. 3. Dtxd immunological identity followed by ELISA. (A) A solution (8 ml) of 9.4 mM Dtxd in PBS containing 37 μM TM® was added to 40 mg of empty PLGA microspheres. The emulsion was incubated, under gentle agitation, during 8 weeks at 37 °C. (B) Control: the 9.4 mM Dtxd in PBS containing 37 μM TM®.

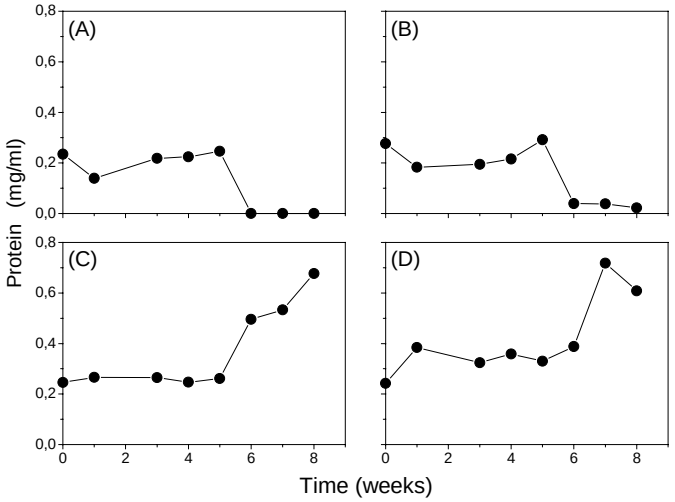


Fig. 4. Kinetics of the interaction of standard Dtxd with empty PLGA microspheres. Dtxd was incubated with PLGA microspheres in PBS pH 7.2 containing 3.7 mM of TM. The supernatants were analyzed by HPLC. (A) Dtxd dimer, (B) Dtxd monomer, (C) Fragment B and (D) Fragment A.

products of this reaction because the absorbance of the formed products (FA-TS and FB-TS) is increased by the overlapping TS absorption signal, we decided to avoid these calculations that always give overestimated results. The peak at 13.356 min corresponds to one of the degradation products of TM (SIBA, SOBA or DTSA).

As a control, the lactic acid solution was added to a pure solution of TM and analyzed by HPLC gel filtration system. Lactic acid changes the solution refractive index and consequently induces changes in the solution absorbance. Here, this phenomenon was exploited in order to follow the reaction between lactic acid and TM. The pure TM peak disappeared,

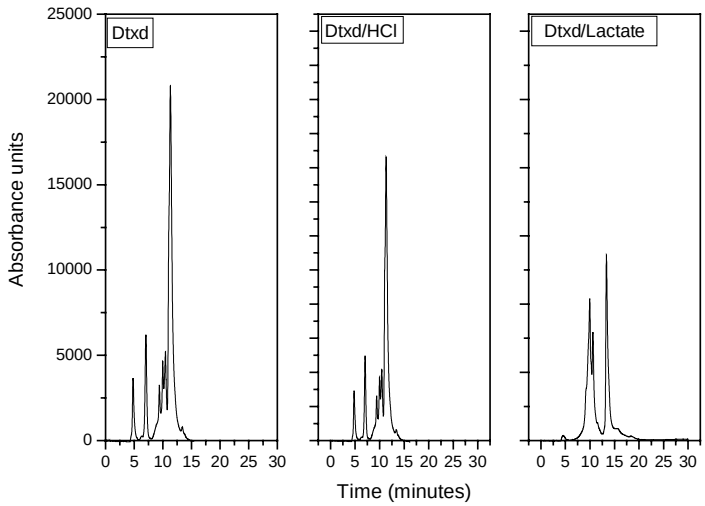


Fig. 5. Elution profile of the Dtxd standard from a gel filtration HPLC column (QC-PAK, GFC 300–7.8 mm × 15 cm) in PBS, pH 7.2 or in lactate containing 3.7 μM TM. Control: HCl in 3.7 μM TM was added to Dtxd standard. Elution flux was 0.6 ml/min, the Rts were TM: 11.58 min, 2× Dtxd: 4.725 min, Dtxd: 7.012 min, FB: 9.872 min, FA: 10.341 min.

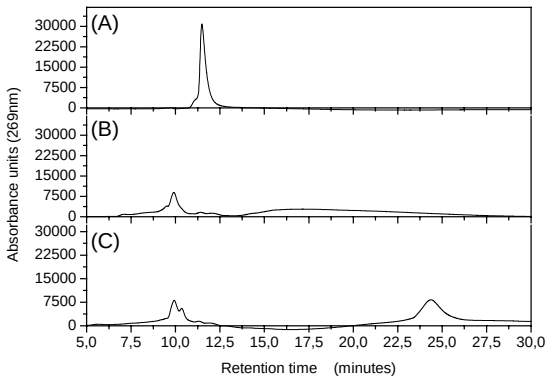


Fig. 6. Interaction between TM and lactic acid. The samples were analysed by gel filtration in a HPLC. (A) TM elution (11.58 min), (B) lactic acid elution (9.89 minutes). (C) incubation mixture (TM and lactic acid) elution, lactic acid (9.89 min), TSA (10.348 min), piruvic acid and EtHg (24.376 min).

and the peak of lactic acid diminished (Fig. 6), indicating that both reacted. TSA is the main product in our general model for Dtxd degradation. It is the only product that has free –SH and cannot be detected in our system because it is highly reactive. The TSA formed was immediately linked to FA or FB. The other products from TM degradations were piruvic acid and EtHg, which eluted at 24.376 min (they were included in the column internal volume).

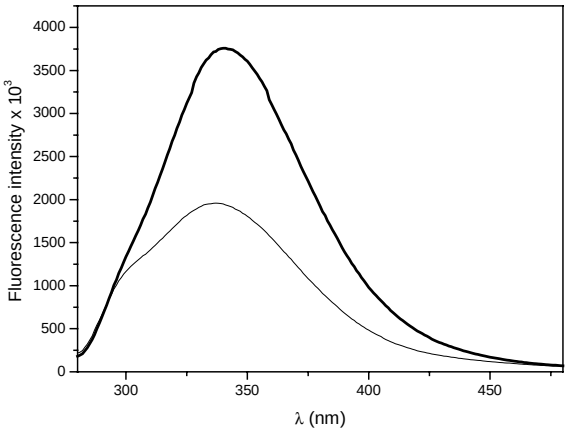


Fig. 7. Effect of lactic acid on the fluorescence spectra of Dtxd. Samples of 1.88 mM Dtxd in PBS containing 37 μM TM were submitted to fluorescence spectroscopy with excitation at 269 nm in the absence (bold line) or in the presence of lactic acid.

By fluorescence analysis, it was observed that lactic acid diminished the fluorescence intensity of the Dtxd in the presence of TM (Fig. 7). This corroborated the ELISA and HPLC data indicating the presence of protein structural damages.

4. Discussion

Thimerosal® is most frequently mentioned as a sulfhydryl reagent, but surprisingly, little attention has been devoted to the mechanism of the reaction between TM and a sulfhydryl compound. The observation, made by several investigators, that the effect of TM is prevented by the sulphydryl compound dithiothreitol (DTT), support the idea that TM exerts its effect via reaction with sulfhydryl groups (Elferink, 1999). It is known that Hg^{2+} may act as a sulphydryl reagent and as an oxidizing agent by reacting with sulphydryl groups like:

$$R–SH + Hg^{2+} \rightarrow R–S–Hg^{+} + H^{+} \quad (1)$$

or

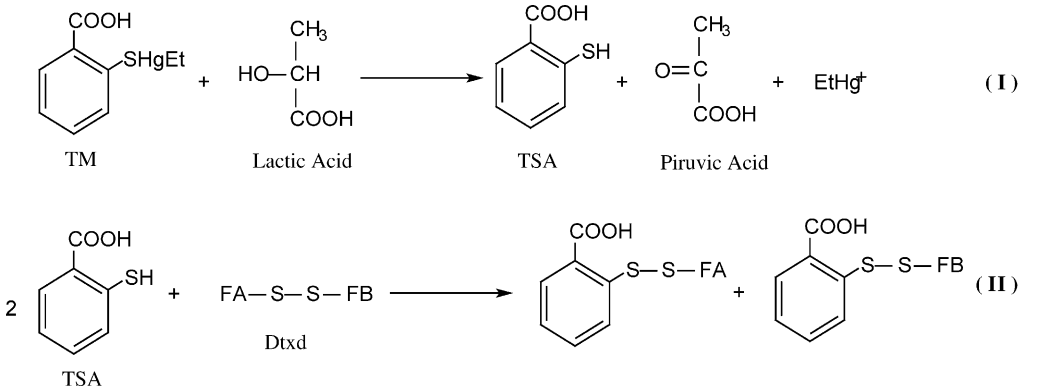
$$R–SH + R–S–Hg^{+} \rightarrow Ar–S–Hg–S–R + H^{+} \quad (2)$$

The reaction of TM with a sulphydryl group of the type R–SH is probably an exchange reaction like:

$$R–SH + Ar–S–Hg–C_2H_5 \leftrightarrow R–S–Hg–C2H5 + Ar–SH \quad (3)$$

The sulphydryl-reactive properties of TM are due to the presence of Hg, because, like other heavy metals, this group has a high affinity for the sulfhydryl group (Elferink, 1999). The Dtxd has no free –SH group, but it has two S–S bonds between Cys-186–Cys-201 and Cys-461–Cys-471. Therefore, it is not plausible that intact TM could react with Dtxd. The Dtxd formulation containing TM is stable in PBS at pH 7.2 for more than 18 months. This is corroborated by data implying that TM did not react with DTNB in vitro (Pintado et al., 1995). Consequently, Dtxd instability (or other protein containing S–S groups) in the presence of TM could be related to the presence of lactic acid in the medium.

It is described in the literature that during TM degradation TSA and DTSA are formed *transiently* because they are absent in degraded samples of TM (Caraballo



Scheme 2. Dtxd reduction mediated by the TSA formed after TM degradation in the presence of lactic acid.

et al., 1993). DTSA, which would be expected to display reasonable stability, is, therefore, reacting with other components in the mixture, the most likely reactant being the EtHg ion (Kang and Schwendeman, 2002) (Scheme 1). By gel filtration on a HPLC system the TM peak (11.58 min) disappeared after reaction with lactic acid (Fig. 6).

The Dtxd damage (Figs. 4 and 5) occurred with a decrease of Dtxd immunological identity, as measured by ELISA (Fig. 3). Dtxd (which contains two S–S groups), after interaction with the PLGA microspheres degradation products, was cleaved in its two fragments, A and B (Figs. 4 and 5), what explains the ELISA data (Fig. 3). The degradation reaction occurred after, at least, 5 weeks of incubation. This behavior is parallel to the lactic acid liberation curve (fifth week) and pH decrease (Fig. 2). As the medium contained TM, we propose that the reduction of Dtxd, followed by oxidation is mediated by TSA (Scheme 2) formed as a degradation product from TM. When, Dtxd was incubated with the HCl no FA or FB increase was observed. Here, the degradation of TM (Scheme 2) we observed is mediated by lactic acid. The TSA produced during this sequence of reactions reduced the Dtxd S–S bridge. The TSA is formed transiently, as described in other systems (Kang and Schwendeman, 2002; Tan and Parkin, 2000 and Caraballo et al., 1993) and it was not detected by HPLC.

It is known that the analogue of EtHg, the ion methyl mercury, is reported not to associate significantly with the disulfide group of cystine (Carty and Malone, 1979) and there are no reports of either methylmercury or EtHg reacting with disulphide compounds.

We concluded that, in aqueous solutions at low pH, with the mandatory presence of lactic acid, the degradation of TM is faster than in neutral media. The media acidification must be promoted specifically by lactic acid. The acidification promoted by HCl did not alter TM (Fig. 5).

We propose that TSA, produced after the reduction of TM by lactic acid, reduces the S–S bridge of the diphtheric toxoid. This reduction is immediately followed by blocking the two –SH formed by the same TSA molecules. In the light of these conclusions it is necessary now, to reinterpret the protein in vitro degradation-stabilization data in the presence of PLGA microspheres containing TM, mainly for those proteins which contain S–S. We recommend that all the PLGA microspheres microencapsulation studies and protein structural considerations should be performed in the absence of TM as a preservative.

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References

Caraballo, I., Rabasco, A.M., Fernandez-Arevalo, M., 1993. Study of Thimerosal® degradation mechanism. Int. J. Pharm. 89, 213–221.

Carty, A.J., Malone, S.F., 1979. In: The biogeochemistry of mercury in the environment. The Chemistry of Mercury in Biological Systems. Elsevier, Amsterdam.

Costa, M.H.B., Fattal, E., 2000. Comparative study of the systemic immune response against a heat shock protein associated to different delivery systems. Proc. Symp. Control. Rel. Bioact. Mater. 27, 6707–6708.

Dogar, V., Sokhey, J., Kumar, S., Sood, D.K., Tahlan, A.K., 2002. Interference of thiomersal in biologicals during protein estimation. Biologicals 30, 271–275.

Elferink, J.G.R., 1999. Thimerosal® a versatile sulfhydryl reagent, calcium mobilizer and cell function-modulating agent. Gen. Pharm. 33, 1–6.

Furr, B.J.A., Utchinson, F.G., 1992. A biodegradable delivery system for peptides: pre-clinical experience with the gonadotrophin-releasing hormone agonist Zoladex. J. Control. Release. 21, 117–128.

Johansen, P., Merkle, H.P., Gander, B., 1998. Physico-chemical and antigenic properties of tetanus and diphtheria toxoids and steps towards improved stability. Biochim. Biophys. Acta 1425, 425–436.

Johnson, O.L., Cleland, J.L., Lee, H.J., Charnis, M., Duenas, E., Jaworowicz, W., Shepard, D., Shahzamani, A., Jones, A.J.S., Putney, S.D., 1996. A month-long effect from a single injection of microencapsulated human growth hormone. Nat. Med. 2, 795–799.

Kang, J., Schwendeman, S.P., 2002. Comparison of the effects of Mg(OH)2 and sucrose on the stability of bovine serum albumin encapsulated in injectable poly(D,L-lactide-co-glycolide) implants. Biomaterials 23, 239–245.

Philippe, M., 1995. The effects of Thimerosal®, a sulfhydryl reagent, on phasic myometrial contractions. Biochem. Biophys. Res. Commun. 211, 1–6.

Pintado, E., Bachero-Leonis, D., Conde, M., Sobrino, F., 1995. Effect of Thimerosal® and other sulphydryl reagents on calcium permeability in thymus lymphocytes. Biochem. Pharmacol. 49, 227–232.

Rappuoli, R., 1997. New and improved vaccines against diphtheria and tetanus. In: Myron M.L., Graeme C.W., James B.K., Gary S.C. (Eds.), New Generations Vaccines, 2nd ed. Marcel Dekker Inc., New York, Basel, pp. 417–436.

Tan, M., Parkin, J.E., 2000. Route of decomposition of thiomersal (Thimerosal®). Int. J. Pharm. 208, 23–34.

Tleugabulova, D., Pérez, I.G., 1996. Reversed phase high-performance liquid chromatographic study of Thimerosal® stability in Cuban recombinant hepatitis B vaccine. J. Control. Release 729, 219–227.

Zhu, G., R, S., Schwendeman, S.P., 2000. Stabilization of proteins encapsulated in injectable poly(D,L-lactide-co-glycolide). Nat. Biotechnol. 18, 52–57.

Zhu, G., Schwendeman, S.P., 2000. Stabilization of proteins encapsulated in cylindrical poly(lactide-co-glycolide) implants: mechanism of stabilization by basic additives. Pharm. Res. 17, 351–357.