Acta Pharm. 53 (2003) 187–197 Original research paper

Biodistribution of ^{131}I-BSA loaded gelatin microspheres after peroral application to BALB/c mice – Particle size study

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Received November 21, 2002
Accepted May 12, 2003

Biodistribution studies of radiolabelled ^{131}I-BSA loaded gelatin microspheres were carried out on BALB/c mice after peroral administration. To two groups, radiolabelled ^{131}I-BSA gelatin microspheres of different particle size, 1.2 ± 1.1 μm and 7.0 ± 1.2 μm, were administered orally. To the control group, a solution of ^{131}I-BSA was administered orally as well. Biodistribution was followed periodically within 15 days as the percent of total radioactivity present in the stomach and small intestine with Peyer's patches and mesentery, in colon with Peyer's patches, appendix and mesentery, in liver, spleen, blood, kidney, lungs and heart. Preliminary *in vitro* biodegradation and drug release studies confirmed the potential of gelatin microspheres to protect the antigen of interest from enzymatic degradation in the gut, and to release it in a controlled manner. The biodistribution data confirmed that particle uptake into Peyer's patches and passage to the liver and spleen *via* the mesentery lymph supply and nodes increased with decreasing particle size.

Keywords: gelatin microspheres, particle size, ^{131}I, bovine serum albumin, biodistribution

A number of potential delivery systems including sustained-antigen releasing microparticulated carriers have been used for targeting Peyer's patches (PP) in order to stimulate particular cells in the gut-associated lymphoid tissue, specifically T helper cells and IgA precursor B cells, for subsequent antigen-specific secretory IgA responses (1, 2). Among these polymeric particles, gelatin microspheres proved to evoke the immune response at the mucosal effector tissues, suggesting the possibility of developing an oral vaccine (3). Due to the many functional groups, proteins could be entrapped in the gelatin matrix by crosslinking with glutaraldehyde and non-covalent bonding forces (4, 5). It has already been shown that emulsification of aqueous solution of gelatin and interferon-α into the particles in oil resulted in high (> 50%) loading efficacy (6), while a loading efficacy higher than 80% was observed for the bovine serum albumin (BSA) (7). The ability of gelatin microspheres to act as regulated and sustained release systems was

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confirmed by subsequent protein release being dependent upon the presence of collagenase (3, 6, 7) and trypsin (8, 9). The rates of gelatin degradation and protein release were inversely related to the extent of glutaraldehyde crosslinking (3, 8) and particle size (7, 9). Research activities of Nakamura *et al*. (3) showed a strong adjuvant effect of gelatin microspheres, confirmed by inducing IgA secretion at the genito-urinary mucosa after peroral application to ddy mice. Particle size employed in this study was in a 2 to 4 μm range.

Although intracellular uptake, intracellular/paracellular uptake and uptake *via* the M-cells and PP are involved in the gastrointestinal uptake of intact particles, the major pathway seems to be uptake *via* the M-cells and PP in the gut (10–12). Further evidence for the importance of PP was provided by Eldridge *et al*. (13) and Jani *et al*. (14). The uptake was restricted to particles below 10 μm in size. The microspheres with 5 to 10 μm diameter remained in the PP, inducing mucosal immunity, while the microspheres below 5 μm in diameter passed through the PP within macrophages, stimulating systemic immunity. Particles of this size were found in the stomach, small intestine with PP appendix and mesentery, liver, blood, kidneys and to a high extent in the spleen 14 days post-administration. These results were confirmed by Ebel (15) who demonstrated that particle uptake depends on the dose level, particle size and the fed state of the animal. Larger particles were not found in the spleen or the mesenteric lymph node. In these conditions, oral administration of vaccines (through their uptake by specialized epithelial cells and subsequent concentration in PP) can induce substantial increase in antigen specific antibodies, secretory IgA in all mucosal sites (gut lamina propria, respiratory tract, mammary glands, salivary glands, lachrymal glands and genito-urinary tract) (1, 2).

Research activities of Araujo *et al.* (16) pointed to the specific biodistribution of polymethyl (2-^{14}C) methacrylate nanoparticles of 130 ± 30 nm diameter administered in a single dose by oral gavage. The nanoparticles were administered in the form of a suspension in saline, in saline with an additional content of surfactants and in oil vehicles. The highest uptake (about 200–300% of the other preparation) was recorded for the saline preparation containing 5% polysorbate 80. There was no significant difference between saline without surfactants and peanut oil, while the addition of oleic acid to the peanut oil increased the nanoparticle uptake by 50%. Radioactivity concentrations were the highest in the GI-tract content and GI-tract walls, decreasing rapidly with time, while the concentration in the residual body was 1–3% of the administered dose at a given time point, remaining at considerable levels for 4 days.

Considering the importance of gelatin microspheres as adjuvants in oral immunization, we prepared BSA-loaded gelatin microspheres with a high drug loading efficacy and particle size ranging from 1 to 7 μm. FTIR-ATR spectroscopic studies have confirmed that, during the preparation process and freeze-drying procedure, only minor conformational changes of protein (α-helix and β-sheet content) were induced, owing to the effective preservation provided by the presence of sorbitol (7, 17). Biodegradation and drug release studies were performed in the presence of collagenase (7). In the present paper, we report biodistribution studies of radiolabelled ^{131}I-BSA loaded gelatin microspheres of different particle size that were carried out after oral application to BALB/c mice. The aim of the work was to evaluate the fate of particles after their uptake into PP, depending on the particle size.

EXPERIMENTAL

Chemicals

The following materials were used for the preparation of microspheres: gelatin from bovine skin (80–110 Bloom) with an isoelectric point (IEP) of 5.1 (Merck, Germany), bovine serum albumin, glutaraldehyde and sorbitan monooleate (SPAN 80) (Sigma Chemical Co., Germany), organic solvents (chloroform, toluene, isopropanol) and sorbitol (Merck). Radiolabelling of BSA was performed by ^{131}I obtained by Biointernational (France). Collagenase (EC 3.4.24.3.) from lyophilized *Clostridium histoliticium* was obtained from Merck.

Preparation of microspheres

The microspheres were prepared by a procedure previously described (7) and originally employed by Tabata *et al.* (6). Gelatin aqueous solution (0.2 mL, 10%, *m/V*) containing 5 mg ^{131}I-BSA was emulsified in a mixture of toluene and chloroform (1:1) containing SPAN 80 in concentrations ranging from 5 to 12% (*m/V*). The resulting emulsion was quickly poured into a precooled mixture of toluene and chloroform (1:3) containing 5% (*m/V*) SPAN 80. The gelatin was crosslinked with glutaraldehyde-saturated toluene (0.05 mg mg^{-1} gelatin) in the emulsion at 0 °C for 4 hours. The resulting microspheres were successively washed with isopropanol, purified water, and then freeze-dried at 200 Pa and –40 °C (Christ α 2–4, Bioblock, Scientific, France) in the presence of sorbitol as a lyoprotectant of protein structure.

Physico-chemical and biopharmaceutical characterization

Microsphere size measurement. – The particle size of the population of gelatin microspheres in aqueous dispersions was determined using laser diffractometry (Particle size analysette D LAB/22, Fritsh, Germany). The spherical form was based on optical micrographs of gelatin microspheres (camera Nikon E-800, Japan) using the phase contrast technique. Particle size was expressed as the mean geometric diameter.

Determination of drug loading. – The content of radiolabelled ^{131}I-BSA entrapped in gelatin matrix was calculated as the percent of total radioactivity present in the supernatant and in the filtrate after microsphere isolation (»well« counter Scaler Type N 529 D, EKCO Electronics, UK).

In vitro *degradation and drug release.* – Gelatin microspheres (0.5 mg mL^{-1}) were suspended in 0.05 mg mL^{-1} calcium chloride containing phosphate buffered saline with collagenase (0.5 mg mL^{-1}). *In vivo* conditions (37 °C, 75 strike min^{-1}) were simulated in a horizontal shaker (Haake SWB 20, Fisons, Germany). At regular time intervals, the dispersion was centrifuged at 8000 rpm for 15 min (Jouan MR 22i Centrifuge, France) and the amount of gelatin in the sediment was determined according to a ninhydrin method that involves determination of α-amino acids, certain amides and amines (18) (Spectrophotometer Lambda 16, Perkin-Elmer, USA).

The amount of BSA released from gelatin microspheres in a presence of collagenase was determined by measuring the radioactivity present in the supernatants at regular

time intervals. In all series of gelatin microspheres, the BSA release profile showed a biphasic modulation characterized by an initial relatively rapid release, followed by a continuous, much slower release that decreased progressively until complete release of BSA.

In order to determine the initial and later time drug release rate, the *in vitro* release profiles were described using the biexponential function: $M_\infty - M_t/M_\infty = Ae^{-\alpha t} + Be^{-\beta t}$, where M_∞ is the total amount of BSA released, M_t is the amount of BSA released at time t, A and B are system-characteristic constants, whereas α and β are rate constants for the initial and later time releases obtained by semilogarithmic plots (19).

Biodistribution studies. – Biodistribution studies were carried out on BALB/c female mice aged 6–8 weeks and weighing about 25 g. Groups of 8–10 mice were housed in an air-conditioned room at 22 ± 3 °C, 55 ± 5% humidity, 12 h light/dark cycles, and maintained on a normal mouse diet. The series of gelatin microspheres with the smallest, 1.2 ± 1.1 μm, and the largest, 7.0 ± 1.2 μm mean diameter were chosen for biodistribution studies in order to evaluate the fate of particles after their uptake into PP, depending on the particle size. Thus, to two groups, 500 μL of saline containing 50 mg ^{131}I-BSA gelatin microspheres of the mean geometric diameter 1.2 ± 1.1 μm (group I) and 7.0 ± 1.2 μm (group II), respectively, were administered orally (intra-gastric administration). To the control group (group III), 500 μL of saline containing 45 mg ^{131}I-BSA (the same as used for preparing the microspheres) was also administered orally. Biodistribution of gelatin microspheres was followed periodically within 15 days (1st, 2nd, 4th, 7th, 9th and 15th day). The animals were sacrificed, the blood was collected, and different organs and tissues were removed. The gastrointestinal tract was separated into the stomach, small intestine and colon. The contents of those parts were collected and the remaining gastrointestinal tract sections were thoroughly rinsed. The radioactivity in the stomach, small intestine with PP and mesentery, colon with PP, appendix and mesentery, liver, spleen, kidney, lungs, heart and blood (2 mL per animal) were determined using a scintillation counter (»well« counter Scaler Type N 529 D, EKCO Electronics, UK) (16). The number of animals (n) per group at a given time point of biodistribution studies was 3. In order to eliminate the radioactivity in the tissues, organs and central circulation due to ^{131}I, only as a consequence of possible iodolysis, preliminary biodistribution studies of ^{131}I--solution were performed after oral administration to group IV of mice. Accumulation of ^{131}I in the thyroid (≈ 99%) at each time point of study (n = 3) was confirmed, so the eventual radioactivity in the tissues, organs and central circulation due to ^{131}I only was eliminated.

RESULTS AND DISCUSSION

Particle size and drug loading efficacy as a function of process parameters

By variations in the process parameters concerning the time of emulsification (from 15 to 30 min) and surfactant concentration (from 5 to 12%, *m/V*) the microspheres with high loading efficacy of 79.8 ± 2.8 to 94.4 ± 2.5% and particle size ranging from 1.2 to 7.0 μm were prepared. Prolonged time of emulsification and increased surfactant concentration led to the formation of particles with a lower mean geometric diameter. The drug encapsulation efficacy followed the microspheres size distribution, so the highest percent of

drug loading was noticed in the series with the lowest value of the mean geometric diameter (Fig. 1).

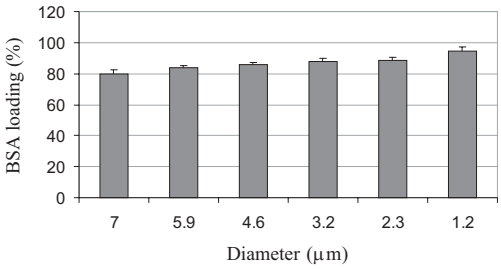


Fig. 1. BSA-loading of gelatin microspheres as a function of the mean particle diameter. Bars denote SD values (n = 5).

Biodegradation and drug release studies of gelatin microspheres

Degradation data of gelatin microspheres showed that the preparation procedure provided prolonged enzymatic degradation in all series of gelatin microspheres, suggesting complete *in vivo* degradation. During 72 h at 37 °C, between 84 and 90% of gelatin microspheres were degraded (7).

After 24 h, the release of BSA from glutaraldehyde treated gelatin microspheres in the presence of collagenase was between 80 and 93% of the total amount of the encapsulated drug. The drug release profiles showed biphasic modulation characterized by an initial, relatively rapid release period of 3 h for particles of 1.2 μm during which 55% of the drug was released, while for the particles of 7.0 μm, nearly 37% of the encapsulated drug was released in the initial period of 6 h (Fig. 2). These data are in correlation with the biodegradation rate, so the higher percent released from the particles with a smaller

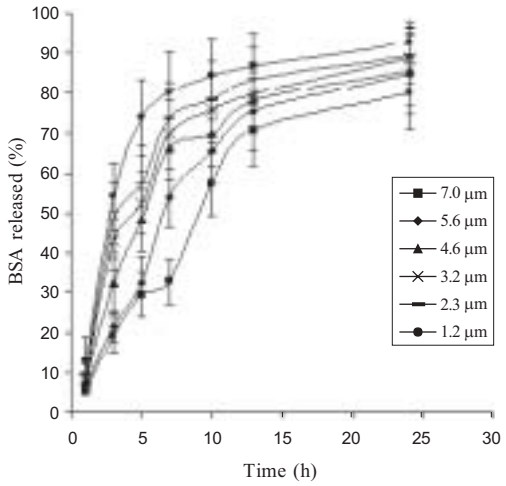


Fig. 2. BSA release profiles from gelatin microspheres of different mean diameters. Bars denote SD values (n = 5).

mean diameter in the initial time release is probably a result of complete water penetration into the spheres supported by faster enzymatic degradation, and thus more free drug liberation.

The drug release constants obtained by the semilogarithmic plots showed that the drug release rate at the initial time ($0.058 < \alpha < 3.21$ h^{-1}) was faster in a series of particles with a smaller diameter, suggesting the influence of degradation and diffusive processes, while the drug liberation in the later release time ($0.066 < \beta < 0.078$ h^{-1}) was influenced by the diffusive processes only (20).

Biodistribution studies

The first day after peroral application of gelatin microspheres with the mean particle diameter of 1.2 μm to group I of BALB/c mice ($n = 3$), 35.0 ± 2.1% of the total radioactivity present in all organs, tissues and blood, was detected in the stomach, small intestine with Peyer's patches and mesentery. The percent of radioactivity appearing in the same region during the 1st day in group II (mean particle diameter 7.0 μm, $n = 3$) was 47.1 ± 3.6% (Fig. 3).

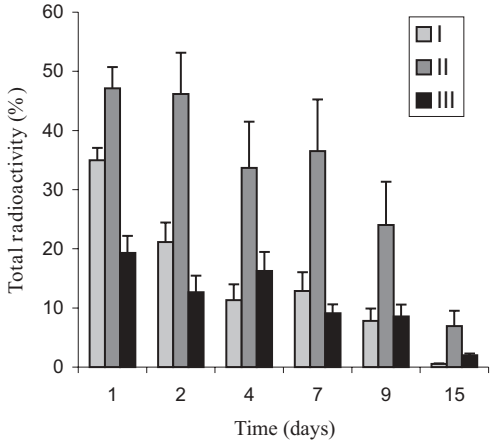


Fig. 3. Biodistribution of ^{131}I-BSA loaded gelatin microspheres with mean diameters of 1.2 μm (group I) and 7.0 μm (group II) and of ^{131}I-BSA solution (group III) after peroral application to BALB/c mice in the stomach and small intestine with PP and mesentery. Bars denote SD values, $n = 3$ at a given point time.

As seen from Fig. 4, total radioactivity present in other organs, separately and in the blood, except in the lungs, was low and relatively similar in the both groups. The low percent of radioactivity can be explained by biodistribution of surface associated and poorly entrapped BSA, and, in addition, BSA released during the »burst« phase, which is susceptible to degradation by digestive enzymes. Also, according to the literature data (13–15, 21), the biodistribution of particles smaller than 5 μm diameter, after being taken up into PP and transported through the efferent lymphatics within macrophages, can contribute to the total radioactivity per organs. Thus, the high radioactivity detected in the lungs in group I (39.2 ± 10.2%) and II (23.9 ± 11.1%) could be explained mainly by the biodistribution of microspheres smaller than 5 μm. Namely, after their uptake into Peyer's patches and passage *via* the mesentery lymph supply and lymph nodes to the

thoracic duct lymph, they traversed through the central circulation to the organs characterized by a dense population of macrophages. The opsonic ability of gelatin of M_ϕ phagocytosis and preferential uptake by macrophages resulted in an increased degradation rate, and thus increased release rate of BSA. The fact that especially dense populations of such cells can normally be found around bronchial lumens explains the high radioactivity present in the lungs during the first 24 h, suggesting the presence of BSA and/or BSA-loaded gelatin microspheres. Research of Iseri *et al*. (22), in which gelatin microspheres were dominantly distributed (> 62%) in the lungs after parenteral application to mice, contributed to the above-mentioned explanation as well.

According to Gebert (23) and Seifert *et al*. (24), an albumin molecule is cleaved fairly rapidly by the digestive enzymes, and the absorption varies in dependence on certain factors, among which is the immune status of the organism. Research of Takahata *et al*. (25) points to rapid degradation of albumin in the presence of trypsin and pepsin, whereas in phosphate buffer saline at 37 °C, after 7 days, albumin was found to be partly hydrolyzed with the prevalence of an antigen fragment at 36.1 kDa. Our research relating to the biodistribution of BSA saline suggests that the radioactivity in tissues, organs and in the central circulation in the control group of mice (group III, n = 3) at a given time point of study could be explained by absorption and distribution/elimination of fragmented BSA (6–9) (Fig. 4).

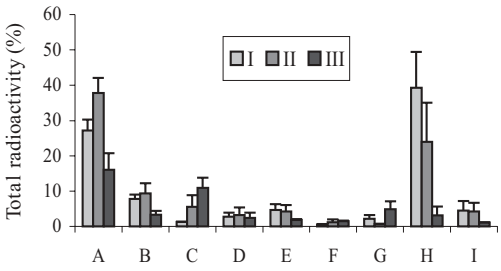


Fig. 4. Biodistribution of ^{131}I-BSA loaded gelatin microspheres with mean diameters of 1.2 μm (group I) and 7.0 μm (group II) and ^{131}I-BSA solution (group III), 24 h after peroral application to BALB/c mice (A – stomach, B – small intestine with PP and mesentery, C – colon with PP, appendix and mesentery, D – liver, E – spleen, F – blood, G – kidney, H – lungs, I – heart). Bars denote SD values (n = 3).

The spleen uptake in group I during 2 days of biodistribution increased to 26.0 ± 6.7%, expressed as the percent of total radioactivity. As the same time in group II, the percent of total radioactivity in the spleen was 8.5 ± 0.9% (Fig. 5). The data referring to the application of radiolabelled BSA-loaded gelatin microspheres in group II suggested fixation of microspheres of the mean diameter of 7 μm at Peyer's patches and mesentery in small intestine. For example, the percent of total radioactivity in this region together with the stomach was 46.1 ± 7.0% in group II *vs*. 21.1 ± 3.3% in group I (Fig. 3).

The data are in accordance with the results of Mathiowitz *et al*. (26): poly-(DL-lactide-co-glycolide) microspheres of a particle size of 1 to 5 μm were microscopically localized in spleen and liver (with apparently normal-looking hepatocytes) 24 h after their application to rats, while 5 to 10 μm microspheres were observed to traverse both the mucosal epithelium through and between individual cells and the follicle-associated epithelium covering the lymphatic elements of Peyer's patches.

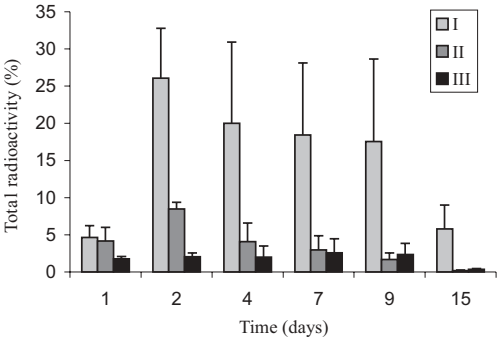
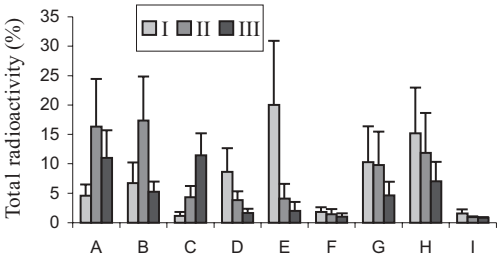


Fig. 5. Biodistribution of ^{131}I-BSA loaded gelatin microspheres with mean diameters of 1.2 μm (group I) and 7.0 μm (group II) and ^{131}I-BSA solution (group III) after peroral application to BALB/c mice in spleen. Bars denote SD values (n = 3 at a given point time).

The same tendency to accumulation of gelatin particles in the small intestine with Peyer's patches and mesentery in group II (Fig. 3), and in the spleen in group I (Fig. 5), was observed also in our experiment on the 4th day of application (Fig. 6). The high radioactivity present in the lungs could be also explained by elimination of gelatin microspheres *via* the lungs where they traverse into the alveoli after being phagocytized by macrophages. After that they travel up the bronchial tree and are then swallowed with the saliva and excreted with the faeces or absorbed from the gut. The radioactivity due to the BSA released from microspheres in macromolecular and digested form cannot be excluded either.

Fig 6. Biodistribution of ^{131}I-BSA loaded gelatin microspheres with mean diameters of 1.2 μm (group I) and 7.0 μm (groups II) and ^{131}I-BSA solution (group III), 4 days after peroral application to BALB/c mice (A – stomach, B – small intestine with PP and mesentery, C – colon with PP, appendix and mesentery, D – liver, E – spleen, F – blood, G – kidney, H – lungs, I – heart). Bars denote SD values (n = 3).



No significant changes in the amount of radioactivity present in the small intestine with Peyer's patches and mesentery in group II (Fig. 3), and spleen in group I (Fig. 5) were noticed on the 7th and the 9th day of administration. The re-absorption of eliminated microspheres (*via* the lungs) from the gut has to be taken into consideration in quantifying the radioactivity in the above-mentioned regions. The significant difference in radioactivity detected in other organs and tissues within individual groups compared to targeted organs and tissues (spleen in group I, and small intestine with Peyer's patches and mesentery in group II) still exists (Figs. 7, 8).

After 15 days of administration, the percent of radioactivity in the regions concerned was up to 5.8 ± 3.2% in the spleen in group I (Fig. 5) and 6.9 ± 2.6% in the small intestine and stomach in group II (Fig. 3), suggesting a low content of microspheres. No radioactivity was detected in other organs and central circulation, except in the lungs

and kidneys, where the radioactivity could be explained by distribution/excretion of microspheres and/or BSA released from the microspheres (Fig. 9).

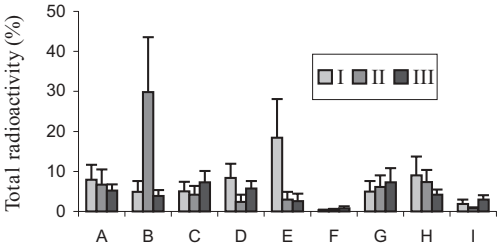


Fig. 7. Biodistribution of ^{131}I-BSA loaded gelatin microspheres with mean diameters of 1.2 μm (group I) and 7.0 μm (group II) and ^{131}I-BSA solution (group III), 7 days after peroral application to BALB/c mice (A – stomach, B – small intestine with PP and mesentery, C – colon with PP, appendix and mesentery, D – liver, E – spleen, F – blood, G – kidney, H – lungs, I – heart). Bars denote SD values (n = 3).

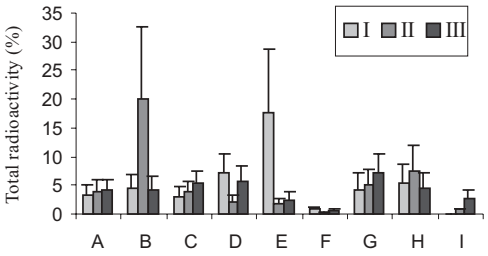


Fig. 8. Biodistribution of ^{131}I-BSA loaded gelatin microspheres with mean diameters of 1.2 μm (group I) and 7.0 μm (group II) and ^{131}I-BSA solution (group III), 9 days after peroral application to BALB/c mice (A – stomach, B – small intestine with PP and mesentery, C – colon with PP, appendix and mesentery, D – liver, E – spleen, F – blood, G – kidney, H – lungs, I – heart). Bars denote SD values (n = 3).

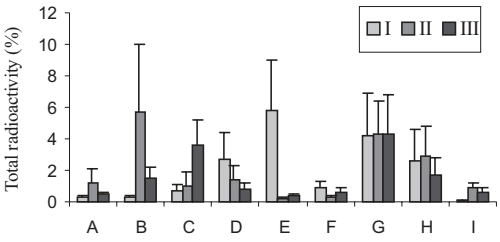


Fig. 9. Biodistribution of ^{131}I-BSA loaded gelatin microspheres with mean diameters of 1.2 μm (group I) and 7.0 μm (group II) and ^{131}I-BSA solution (group III), 15 days after peroral application to BALB/c mice (A – stomach, B – small intestine with PP and mesentery, C – colon with PP, appendix and mesentery, D – liver, E – spleen, F – blood, G – kidney, H – lungs, I – heart). Bars denote SD values (n = 3).

CONCLUSIONS

Due to the existence of common mucosal immune system, successful targeting of vaccines to inductive compartments within gut-associated lymphoid tissues may induce local humoral responses in lymphoid tissues at many mucosal loci. The ability to protect mucosal surfaces, in other words, the common sites of pathogen entry, is perhaps the greatest attribute of oral approach to immunization. Biodegradation studies of crosslinked gelatin microspheres confirmed their stability to digestive enzymes, such as collagenase, in the gastrointestinal tract. According to the drug release studies, an initial pulse of antigen followed by continuous delivery can provide protective and lasting im-

munity. Particle size appears to be a key parameter. Gelatin microspheres of 1 μm showed great promise of inducing a predominantly systemic antibody response owing to their propensity to translocate to systemic lymphoid tissues, such as spleen. Orally administered gelatin microspheres of a 7 μm diameter tend to be adsorbed through the M cells into Peyer's patches, thus providing a higher local antigen concentration with a potential to elicit local responses within this mucosal immune-inductive site. In the light of the above-mentioned facts, our biodistribution studies showed a great promise of gelatin microspheres as adjuvants in oral vaccine.

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S A Ž E T A K

Biodistribucija želatinskih mikrosfera s ^{131}I-BSA poslije peroralne primjene kod BALB/c miševa – Utjecaj veličine čestica

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Praćena je biodistribucija želatinskih mikrosfera s ^{131}I-BSA i različitom veličinom čestica poslije peroralne primene kod BALB/c miševa. Biodistribucija je praćena u različitim vremenskim intervalima tijekom 15 dana kao postotak ukupne radioaktivnosti prisutne u želucu i tankom crijevu s Pajerovim pločama i mezenterijumom, u kolonu s Pajerovim pločama, apendiksom i mezenterijumom, te u jetri, slezeni, bubrezima, plućima, srcu i krvotoku. Rezultati ispitivanja ukazuju na utjecaj veličine čestica u lokalizaciji i preuzimanju u Pajerovim pločama i raspodjeli u jetri i slezeni preko mezenterične limfne cirkulacije.

Ključne riječi: želatinske mikrosfere, veličina čestica, ^{131}I, goveđi serumski albumin, biodistribucija

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