HHS Public Access

Author manuscript

Adv Healthc Mater. Author manuscript; available in PMC 2017 March 01.

Published in final edited form as:

Adv Healthc Mater. 2016 March ; 5(6): 653–658. doi:10.1002/adhm.201500797.

Particles for Local Delivery of Proteins Using Intra-Articular Route

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Abstract

Designing a vehicle for local delivery of proteins using intra-articular route is an attractive option to minimize the adverse effects associated with systemic exposure and to maximize the efficacy. Slowly-dissolving silylated micro-particles are designed with specific size and shape that are capable of extending the retention time of a model protein (BSA) in the murine knee joint. No cytotoxicity is observed for the reconstituted formulation when tested against synovial fibroblasts and RAW macrophages.

Graphical Abstract

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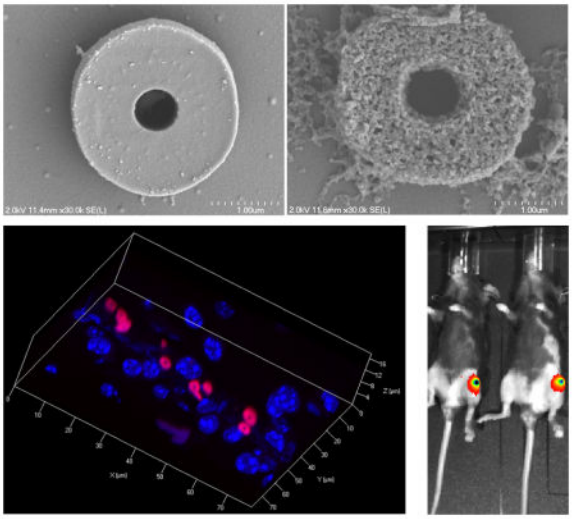
Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.



Keywords

micro-particles; intra-articular; bovine serum albumin; silylation; joint retention

Local treatment of arthritis is an attractive option when the symptoms are restricted to individual joints. However, benefits of local treatment can be limited by poor retention of the drug in the joint.[1] The principal advantage of local delivery is that only a minimal amount of drug is required to exert the desired pharmacological activity thereby reducing drug exposure to inappropriate sites.[2–4] Furthermore, due to lack of vascularity in articular cartilage, regional administration is more beneficial compared to administration into the systemic circulation.[5] Specifically, for protein based drugs with low bioavailability by other routes, regional administration through the intra-articular (i.a.) route is a valuable option.[6] It is known that entry of macromolecules into the synovial fluid from systemic circulation is inversely proportional to their molecular weight,[4] and therefore the higher the molecular weight of the therapeutic, the more difficult it is to systematically deliver it to the joint. According to Mitragotri and Yoo,[7] carrier systems that release the drug in a controlled manner in the synovial area and are rationally designed based on physiochemical properties of the drug and pathophysiological characteristics of the disease are greatly needed for treating arthritis. In this work, possibility of using slowly-dissolving silylated bovine serum albumin (BSA) particles (functionalization and characterization details can be found elsewhere),[8] as a carrier for i.a. delivery is investigated. We demonstrate that these silylated particles can be lyophilized in a non-aqueous mixture to confer physiochemical stability to the formulation. Cell viability study of murine macrophages and synovial fibroblasts [9] confirm that functionalization does not induce cytotoxicity in the formulation. Upon reconstitution of the lyophilized formulation, particles are then delivered to the mouse knee joint using i.a. route to determine their *in vivo* retention.

Half-lives ($t_{1/2}$) of soluble proteins in the joint of different animal models have been extensively studied. Larsen et al.[2] compiled a list for $t_{1/2}$ of various molecules in joints of non-murine models. Clearance of native BSA from rabbit joint is relatively fast with $t_{1/2}$ of

only 3.91 hours (hr).[10] Based on the experiments of van den Berg and van de Putte,[11] negatively charged native BSA shows no affinity for cartilage and high amounts of antibodies are needed for its retention in the mice joint. On the contrary, the retention of cationic BSA in non-immune mice joint is very high. Moreover, chronic joint inflammation in immune mice (antigen + Freund's complete adjuvant (FCA) injected into flank skin and the footpads of the forelegs + intraperitoneal injection of heat-killed Bordetella Pertussis organisms as an additional adjuvant) only developed after i.a. administration of cationic antigens (methylated BSA, amidated BSA) but not native BSA.[12] For immune mice (BSA +FCA) even with the administration of a booster (BSA+FCA), the retention of native BSA in the joint is still low. Experiments of van Lent et al.[13] with radiolabeled proteins in mice confirmed that both charge and size of the protein are important factors for determining the penetration and localization in the joint. Moreover, upon i.a. injection of radiolabeled native human immunoglobulin G (IgG) and BSA, less than 1% of the injected dose can be detected in the joint after 24 hr. Lymphatic drainage is expected to be the primary mechanism responsible for the clearance of soluble form of proteins from the joint cavity,[4,14–16] and experimentally it has been observed that the clearance times of anionic native proteins with disparate molecular weights (IgG and BSA) are of the same order.[12–14]

Several researchers have used particulate based carrier systems with variety of sizes and compositions to investigate their potential in increasing the joint retention time or reducing inflammation. Horisawa et al.[17] have studied the size effect of particles made from poly (D,L-lactide-co-glycolide) (PLGA) in rats by comparing the retention of 265 nm and 26.5 μm particles. Liggins et al.[18] prepared paclitaxel loaded PLGA, poly (L-lactic acid), and poly (caprolactone) with different sizes to investigate biocompatibility and efficacy in rabbits for treatment of inflammation. Butoescu et al.[19] have used magnetic PLGA nano-particles for delivery of dexamethasone 21-acetate to the mouse joint. Nishide et al.[20] have studied *in vivo* fate of micro-spheres made of D,L-lactic acid oligomers of different molecular weights and sizes. Brown et al.[21] have tested gelatin plus chondroitin-6-sulfate micro-spheres for delivery of labeled catalase and albumin into mouse joint. Block copolymers have been used to tether interleukin-1 receptor antagonist (IL-1Ra) to the particles for joint retention study in rats.[22] Inoue et al.[23] measured the residual dose of basic fibroblast growth factor contained in glutaraldehyde (GA) crosslinked gelatin micro-spheres.

Review by Gerwin et al.[24] gives an in-depth view on the i.a. delivery route including the micro and nano-particulate systems. Beside the polymeric and gelatin based systems, albumin particles have been used as a potential carrier for delivery of therapeutics. The advantage of albumin particulate system is lack of toxicity and biodegradation into natural products.[25] Human serum albumin (HSA) has been used as a vehicle for targeted delivery of methotrexate.[26] Heat-stabilized (150 °C for 40 hr) rabbit serum albumin (RSA) micro-spheres have been prepared for delivery of steroids with $t_{1/2}$ of 62.5 hr.[27] BSA has also been studied as a viable injectable biodegradable system for sustained release of progesterone in rabbits,[28] and as a matrix for delivery of dexamethasone,[29] diclofenac sodium,[30] and naproxen sodium.[31] Ratcliffe et al.[32] intra-articularly delivered radio-labeled RSA using GA crosslinked micro-spheres and noticed $t_{1/2}$ of 3 days. The authors also observed that particles made from RSA cause minimal histological change in rabbit

synovium and thus are good candidates for a biocompatible delivery vehicle.[33] Two major problems with GA crosslinked and heat stabilized albumin particles are formation of uncleavable crosslinks and loss of molecular secondary structure upon denaturation which proves to be detrimental to the protein functionality. Moreover, the shapes of these fabricated particles are restricted to simple and basic geometries like spheres. Size and shape of the particles can both play important roles in triggering an inflammatory reaction and it has been demonstrated that irregularly shaped particles promote tissue inflammation when compared to round-shaped objects.[6]

This communication focuses on developing a particulate albumin system for delivering proteins using i.a. route by rectifying the above-mentioned shortcomings, and then compares the joint retention of the slowly-dissolving particles with that of a protein solution. Stability is conferred to the particles with diisopropyldichlorosilane (DIDCS) as a crosslinking agent that retains the secondary structure of the molecule while shape and size of the particles is precisely controlled with Particle Replication in Non-wetting Templates (PRINT) to prepare micro-toroids with rectangular cross-section.[8]

Upon fabricating micro-particles containing 55.7 wt % BSA (model protein), 43.1 wt % lactose, and 1.2 wt % glycerol with PRINT platform, they are functionalized with DIDCS to make them slowly-dissolving in aqueous medium. DIDCS which belongs to the family of dichlorosilanes reacts with BSA and glycerol to make the particles slowly dissolving by introducing a silyl group in place of a labile hydrogen atom and combining molecules together. The scanning electron microscopy (SEM) images of Alexa Fluor 680 conjugated BSA loaded micro-particles are shown in Figure 1.A and 1.B. To confer storage stability and prepare a reconstitutable powder, particles are freeze-dried from a mixture of poly (vinyl pyrrolidone) (PVP) and tert-butanol (TBA). According to Cingolani et al.[34] TBA can stabilize proteins by decreasing their solubility and inducing salting-out and has been used in pure form by Ni et al.[35] for freeze-drying of an antitumor drug. Use of TBA allows us to prepare stable dry particles with minimal aggregation that are not exposed to water prior to reconstitution. Furthermore, addition of PVP prevents the aggregation of the particles during the lyophilization stage by acting as a bulking agent (Figure 1.C). These particles (loaded with Alexa Fluor 555 conjugated BSA) can then be uniformly dispersed in the reconstitution media composed of PBS+0.1% w/w Tween 80 (Figure 1.D and 1.E). The rational for using Tween 80 in the formulation is to improve the dispersibility of the particles and is based on its low cytotoxicity.[36] Functionalized particles freeze-dried in TBA+5% w/v PVP (Figure 1.F) retain their shape after a hydration/dehydration cycle of an environmental SEM (Figure 1.G) with PVP easily dissolved to recover the individual particles.

The longer the reaction (rx) time of the particles with DIDCS, the slower is their dissolution rate.[8] In Figure 2.A–2.H effect of exposure of the particles to the reconstitution medium post lyophilization from freeze-drying medium (TBA+5% w/v PVP) is evaluated with SEM. The 24 hr silylated particles are highly stable in aqueous medium with very slow dissolution rate. After spending 4 days in reconstitution medium, the surface morphology of this set of particles changes (Figure 2.C and 5.G.) and then 4 days later they are very soft and deformable (Figure 2.D and 2.H). However, when the particles are silylated for only 12 hr, even a brief exposure (10 minutes) to the reconstitution medium results in severe structural

changes (Figure 2.B and 2.F). Unfunctionalized dry particles that were not exposed to the reconstitution medium are also shown for comparison (Figure 2.A and 2.E). These observations are consistent with the faster dissolution rate of 12 hr silylated particles when compared to the particles silylated for 24 hr,[8] and qualitatively show the structural changes associated with the exposure of the silylated particles to the reconstitution medium. The relative size of the particles is compared with the diameter of the needle used for the i.a. injections in Figure 2.I–2.K.

Activated synovial fibroblasts play an important role in arthritis,[37] and it is known that synovial fibroblasts are responsible for particle ingestion and uptake after i.a. delivery.[38,39] Brown et al.[21] evaluated the cytotoxicity of their particles against synovial fibroblasts extracted from arthritic patients. RAW macrophages have also been used to evaluate the cytotoxicity of nano-particles for i.a. delivery.[22] Viability of the cultured (flow cytometry data in Figure S1) synovial fibroblasts (Figure 2.L) and RAW macrophages (Figure 2.M) were determined, once they were incubated with different combination of ingredients used in preparation of the injectable formulation (data shown in Figure S2). Figure 2.L, 2.M show the results for PVP and Tween 80 which serve as a measure for evaluating the inherent cytotoxicity of the reconstitution medium. In the case of synovial fibroblasts, no cytotoxicity for unfunctionalized particles could be detected whereas there is a reduction of viability for RAW macrophages (Figure 2.M). This reduction might be attributed to the nitric oxide production due to stimulation of macrophages by BSA.[40] No reduction of viability was observed for functionalized particles.

Upon optimizing the i.a. injection (Figure S3), Alexa Fluor 680 conjugated BSA loaded particles (Table S1) were intra-articularly delivered to the knee joint of mice. Figure 3 juxtaposes the time course clearance of the functionalized particles with that of the soluble form (unfunctionalized) for the group of animals studied (Figure S4). The rate at which the radiant efficiency (calibrated in Figure S5) and fractional fluorescence remaining in the joint diminish is notably faster for the unmodified form compared to the functionalized particles.

The areas under the curve for 24 hr silylated and unmodified particles (Figure S6) were calculated as 5.16E+9 and 2.91E+9 [p/s/cm^2/sr][hr]/[μW/cm^2], respectively. Furthermore, the half-lives of these samples were determined to be 19.4 and 4.4 hr. Figure 2.N–2.Q histologically evaluate the Alexa Fluor 680 conjugated BSA particles in the joint 12 hr post injection upon isolation of the knee (Figure S7). Particles (red) could be located in different positions inside the joint cavity 12 and 36 hr after injection (Figure S8 and S9). Moreover, infiltration of cellular components (blue) stained with DAPI (4′,6-diamidino-2-phenylindole) in the joint cavity can be seen in Figure 2.P and 2.Q in the close proximity of the particle aggregates. These figures suggest that some of the particles remain in the synovial cavity while slowly dissolving (releasing their protein content) and some are associated with the synovial membrane and thus cleared throughout the course of our *in vivo* experiment. Figure 2.O and Figure 2.Q are confocal images obtained from 3D rotation of Figure 2.N and 2.P, respectively. (Video S1 and S2)

In this communication, we demonstrated that the dissolution of the silylated particles is accompanied by surface morphological changes as observed with SEM. Cell viability

studies on RAW macrophages and synovial fibroblasts confirmed that the functionalization of particles does not attribute cytotoxicity to the particles. Half-life of the fluorescent labeled BSA after incorporation into the 24 hr silylated particles is 4.4 times and the averaged area under curve for fluorescent signal versus time in the joint is 1.78±0.39 times of that of the unmodified soluble protein. Although Rodnan and Maclachlan,[14] did not observe any differences between the rates of clearance of heterologous albumin (HSA) and homologous albumin (RSA) in normal rabbit joint, fabricating particles from homologous albumin is recommended to safely neglect the antigenic cause of clearance. Immunogenicity and charge state of the silylated BSA used as a carrier in the particle composition is an important factor when preparing an injectable formulation for arthritis and should be diligently studied. According to Lee et al.[28] injectable beads prepared by crosslinking BSA under mild conditions using GA should yield a non-immunogenic and biodegradable device for drug delivery. Silylation of BSA particles may change or create new epitopes on the protein, which can induce antibodies against the particle carriers and result in fast clearance of carriers. However, our imaging data suggest that the particles are retained in joint space for a much longer time than soluble BSA. Although we do not expect generation of antibodies against homologous albumin, a full evaluation of the immunogenicity of the particles post silylation in future will help us better understand the properties of this system. Charge state determination by isoelectric focusing helps elucidate the charge interaction of the particles and released BSA molecules with the articular cartilage.[12]

Potential use of di-tert-butyldichlorosilane (with bulkier side groups) in extending the retention time of the particles needs to be further investigated.[41] Whereas, it is beyond the scope of this communication, PRINT technology proves to be helpful for systematically elucidating the role of shape and size in retention/clearance of the particles from the joint. Flexibility of PRINT technology to engineer non-spherical geometries has been discussed elsewhere.[42,43] Other BSA containing particles prepared with PRINT platform can be used to investigate the effect of shape on particle retention (Figure S10).

Experimental Section

The detailed experimental procedures are available in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge the National Institutes of Health (NIH) Director’s Pioneer Award and Liquidia Technologies for support. Joseph M. DeSimone is the founder and maintains a financial interest in Liquidia Technologies. All the animal studies were approved by Institutional Animal Care and Use Committee (IACUC) of University of North Carolina at Chapel Hill (UNC-CH). Microscopic evaluations were done at Microscopy Service Laboratory (MSL) and histological slides were prepared at Animal Histopathology core facility of UNC-CH.

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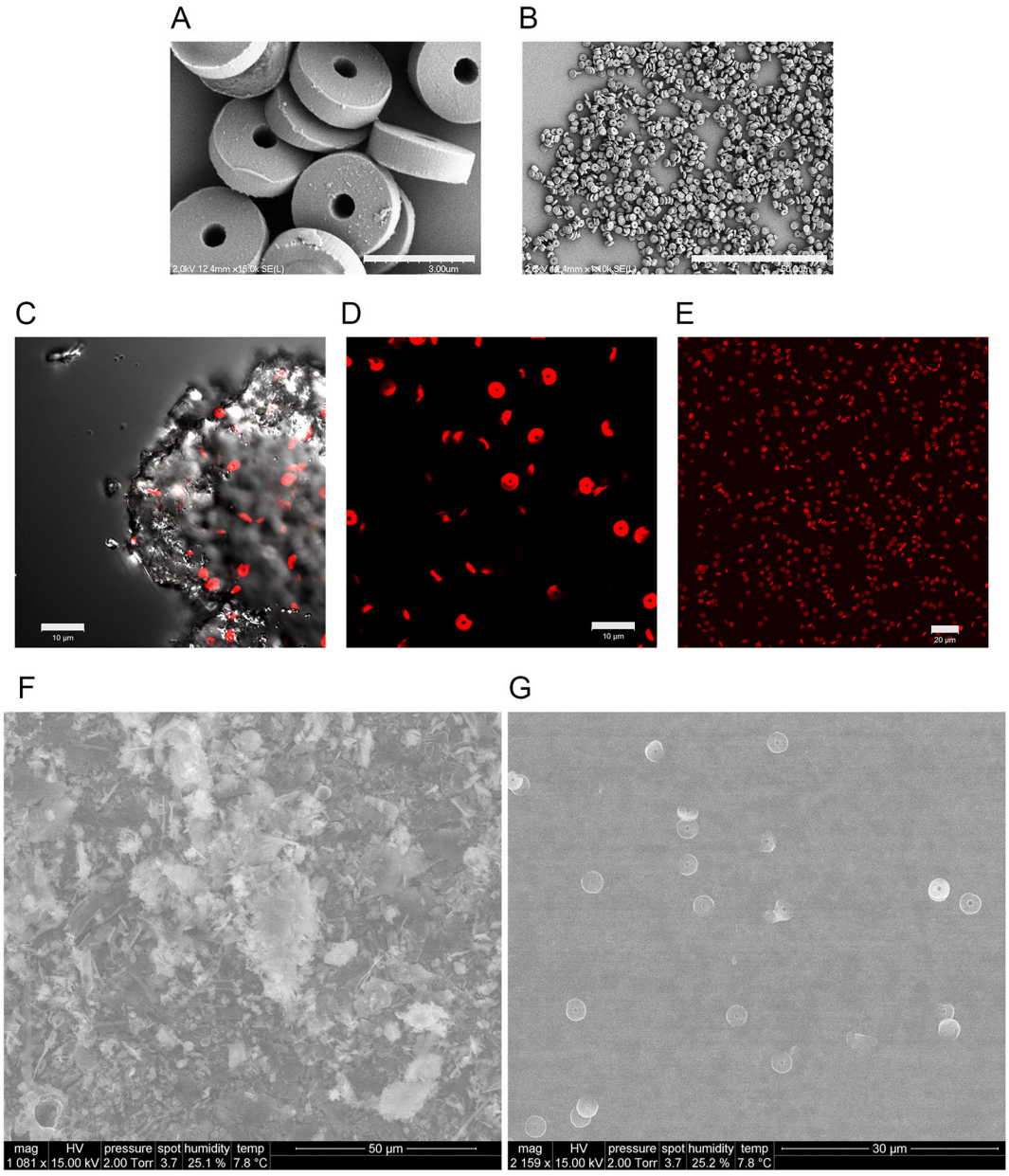

Figure 1.

Particles fabricated with PRINT platform at two different magnifications. A.×15k-scale bar = 3.00 µm. B.×1.1k-scale bar = 50.0 µm. C. Dry cake of 24 hr functionalized particles freeze dried from 5% w/v PVP-TBA-scale bar = 10 µm. D. particles dispersed in 0.1% w/w Tween 80 in PBS (high magnification)-scale bar = 10 µm. E. particles dispersed in 0.1% w/w Tween 80 in PBS (low magnification)-scale bar = 20 µm. (Excitation: 543 nm, Long pass filter: 560 nm) Hydration cycle effect studied with Environmental SEM. F. Particles freeze dried in 5% PVP+TBA prior to cycle-scale bar = 50 µm. G. 24 hr silylated particles freeze dried in 5% w/v PVP+TBA after the cycle-scale bar = 30 µm.

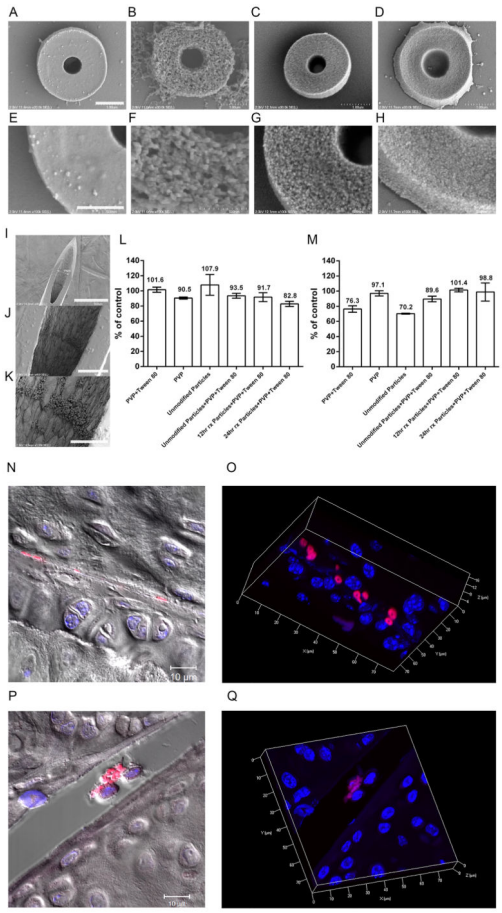

Figure 2.

An unfunctionalized particle not exposed to PBS (A.×30k magnification-scale bar = 1.00 μm, E.×100k magnification-scale bar = 500 nm). Silylated for 12 hr and exposed to PBS +0.1% w/w Tween 80 for 10 minutes (B.×30k magnification, F.×100k magnification). Silylated for 24 hr and exposed to PBS+0.1% w/w Tween 80 for 4 days (C.×30k magnification, G.×100k magnification). Silylated for 24 hr and exposed to PBS+0.1% w/w Tween 80 for 8 days (D.×30k magnification, H.×100k magnification). Relative size of the particles compared to 30G ½ needle (176 μm in diameter) used for i.a. injections (I.×90-scale bar = 500 μm J.×400-scale bar = 100 μm K.×1k-scale bar = 50.0 μm) Percent cell viability of different compositions at 1 mg/mL for 12 and 24 hr silylated samples (L. synovial fibroblasts, M. RAW macrophages). Functionalized Alexa Fluor 680 conjugated BSA loaded particles 12 hr post injection into the murine knee joint cavity. N. Red+Blue +DIC-scale bar = 10 μm. O. Red+Blue, 3D confocal rotation. Presence of cellular components in the joint cavity P. Red+Blue+DIC-scale bar = 10 μm. Q. Red+Blue, 3D confocal rotation. (Red: Alexa Fluor 680 conjugated BSA Excitation: 639 nm Long pass filter: 640 nm, Blue: DAPI Excitation: 405 nm, Band pass filter: 420–475 nm + 500–610 nm, DIC: Differential Interference Contrast, rx: reaction)

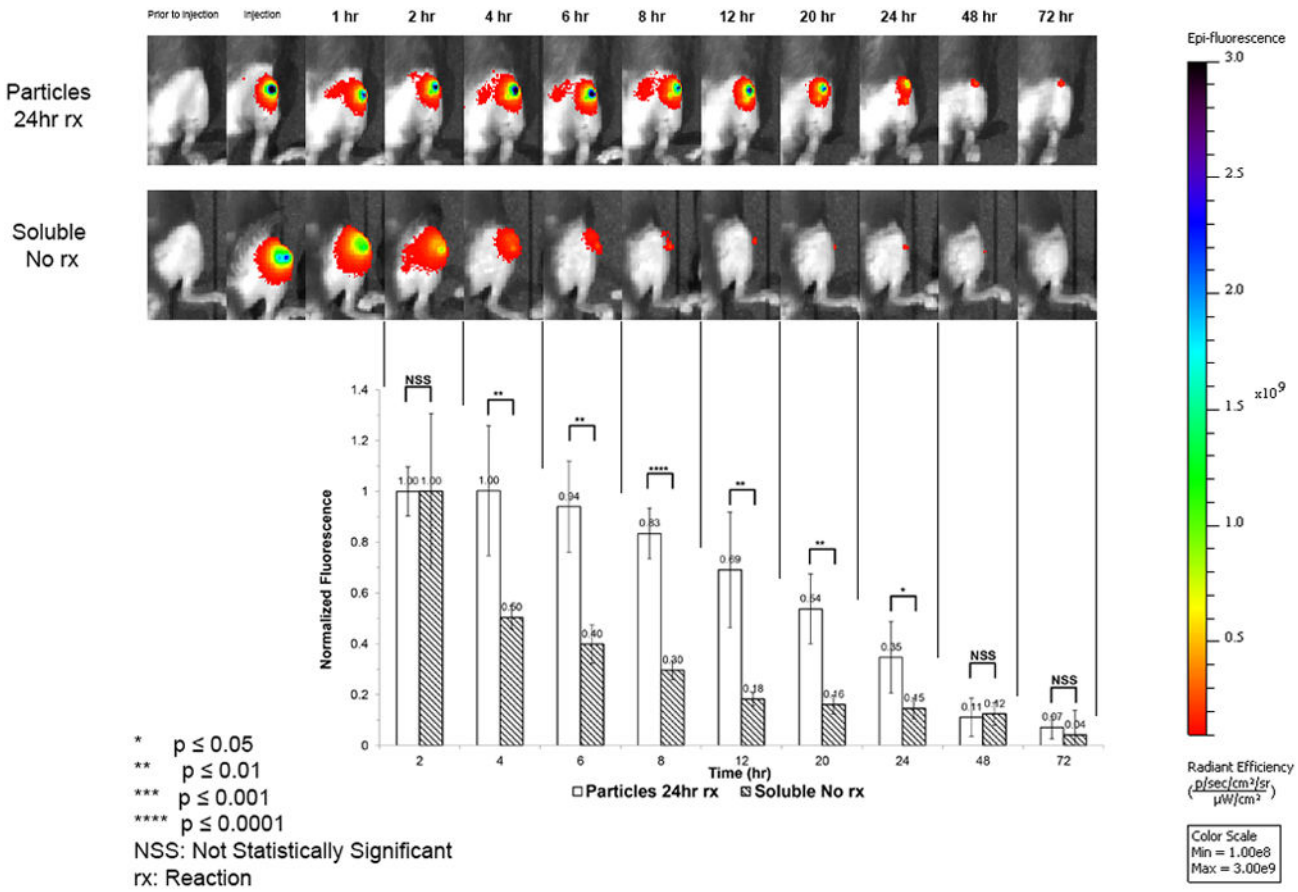

Figure 3.

Time course clearance of the functionalized particles containing Alexa Fluor 680 conjugated BSA compared to the intact particulate formulation at 2, 4, 6, 8, 12, 20, 24, 48, and 72 hr post injection as measured by radiant efficiency emanating from the knee joint. (n=4, rx: reaction)