Design and Delivery of Silver Sulfadiazine From Alginate Microspheres-Impregnated Collagen Scaffold

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Abstract: A reconstituted collagen scaffold impregnated with silver sulfadiazine (SSD) loaded alginate microspheres, capable of delivering the drug in a controlled manner has been developed. SSD-loaded alginate microspheres were prepared by modified water-in-oil emulsion technique through interfacial ionic gelation of alginate using $CaCl_2$. The SSD-loaded microspheres were impregnated in pepsin-solubilized collagen, *in situ*, while inducing fibrillation and cast as thin scaffold. Morphological features of microspheres and microsphere-impregnated collagen were analyzed through SEM. Distribution homogeneity of impregnated microspheres, their *in vitro* behavior in (Dulbecco's modified minimal essential media) DMEM, and antibacterial efficiency against *ATCC* pathogens were determined. Initial drug load of 20% (w/w) with respect to alginate and 40% (v/v) of 2% alginate with respect to oil phase were found to produce microspheres of optimum drug entrapment (3%) and required size range (300–370 μm). *In vitro* drug release studies from the scaffold showed an initial burst release of 47.5% and a controlled release for 72 h with equilibrium concentration of 68.8%. SSD-loaded microspheres exhibited minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) levels of 32 and 40.2 μg/mL to both *K. pneumoniae* and *E. coli* respectively. *P. aeruginosa* showed MIC and MBC levels of 44.8 and 51.2 μg/mL respectively, while *Staphylococcus aureus* exhibited MIC and MBC at the same concentration range (57.6 μg/mL). The collagen-based scaffold impregnated with SSD-loaded alginate microspheres can deliver SSD in a controlled fashion, can control infection for extended time period with lesser dressing frequencies, and will enable easier assessment of wound. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 77B: 378–388, 2006

Keywords: silver sulfadiazine; alginate microspheres; ionic gelation; collagen scaffold; controlled release

INTRODUCTION

An ideal wound dressing is one, which induces host cells to regenerate, prevent infection, act like an osmotic regulator,[1] providing optimal environment for healing to take place quickly.[2] Although occlusive dressings possess clinical benefit in treatments of wounds,[3,4] they may provide a favorable environment for the microflora of wound to further proliferate and grow.[5] Microbes, entering the wound, delay healing by initiating infection, leading to wound fluid exudation, and have a number of deleterious effects on the healing process.[6,7] The bacterial colonies may induce scarring, resulting in wound dehiscence, reduction of oxygen tension, and destruction of existing tissue matrix.[8,9] Still, less attention has been given to the contribution of dressings capable of controlling the clinical outcome.

Among the recently available dressings, collagen-based dressings have shown to induce accelerated wound healing.[10] Collagen is well known for its wide biomedical application potential. Though many natural biomaterials are available, collagen stands apart from them, mainly because of its mode of interaction with the host tissue.[11] Especially, collagen films, by virtue of their capability to regulate release profiles of incorporated drugs, have been widely used as carrier constructs.[12–14] Apart from that, as described earlier, collagen scaffolds possess high biocompatibility and physicochemical properties, including essential tensile strength.[15] Hence collagen-based scaffolds can be used as a potential system for delivering drugs. Still, only a few collagen-based dressings incorporated with active therapeutic agents capable of controlling infection during healing process are developed.

The best-identified topical antibacterial agent to control infections experimentally and clinically is silver sulphadiazine (SSD).[16,17] It possesses a broad spectrum of activity against gram-positive and gram-negative bacteria as well as fungi.[18–20] Its ability to be effective even at low concentra-

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tion in reducing invasive early burn wound sepsis[21] has made it a drug of choice for burn wound injuries. Pre-clinical and clinical data pertaining to its action towards control of burn wound infection have been reported with reference to the usage of commercially available creams.[22–25]

A major cause of concern during usage of SSD cream is absorption of silver ions, which remain at a certain level in the body for some time after dissociation.[26] Earlier studies concerned with absorption of silver ions have shown that most silver is associated with superficial eschar and very little is absorbed.[21] Later it was proved that significant absorption occurs in large burns.[27,28] Because of this reason usage of cream is being avoided in treating second-degree burns. Moreover, optimal dose and mode of deployment of currently available SSD cream in burn wound therapy have not been fully defined. Solution to this problem and proper defining of these would provide a better way of controlling sepsis.[29]

Alternative systems have been developed to overcome the aforementioned problems.[30,31] But these systems lack the ability to control the release of SSD in such a way that drug concentration in serum does not exceed toxic levels when used for *in vivo* application. A possible method of controlling the delivery of SSD is by entrapping it in a polymer matrix. Among the polymers, sodium alginate, an anionic polymer of 1,4-linked β-D-mannuronic acid and α-L-guluronic acid, has been extensively used for the development of carriers. Alginate microcarriers can be prepared easily in aqueous solution at room temperature without involvement of any harsh conditions. It possesses immense swelling property, which increases the contact time of alginate carrier and the layer over which it is applied.[32,33] Alginate-based microcarriers are used in the development of several delivery systems, such as oral, nasal systems, etc.[34,35] These characteristic features of alginate provide enormous scope to use it as an effective carrier material.

Though control of burn wound bacteria is given an overriding importance, the absorption of silver through burn wounds treated with SSD is a source of rising concern and prompted us to develop a collagen-based system, which can actively involve in the healing process as well as prevent infection. Hence this work describes the development and characterization of a reconstituted collagen scaffold impregnated with SSD-loaded alginate microspheres, capable of delivering SSD in a controlled manner. This would be a probable solution to effectively manage burn wound infection, with active participation of wound dressing in healing process, and lessen the cause of concern towards silver toxicity and frequency of routine dressing change.

MATERIALS AND METHODS

Materials

Silver sulfadiazine was obtained from Aldrich, USA. Pepsin and Dulbecco's modified minimal essential media (DMEM) were obtained from Sigma, USA. Sodium alginate (medium viscosity, 3500 cps) was obtained from Sigma, USA and Span-80 was obtained from Fluka, Switzerland. *n*-Octanol was procured from Merck, Germany. All microbiological media were obtained from HIMEDIA, Mumbai, India. All other chemicals used in this work were of analytical grade.

Methods

Preparation of Alginate Microspheres. Sodium alginate microspheres were prepared by water in oil (w/o) emulsification and subsequent ionic gelation technique employing the method previously described[35] with modifications. In detail, a w/o emulsion was prepared by slowly adding 40 mL of 2% (w/v) of sodium alginate to 100 mL of *n*-octanol containing 5% span 80 as emulsifier, and the mixture was sonicated (Vibracell, viewsonics 600 Hz, USA) for 15 min under constant amplitude of 42% with intermittent on/off pulse of 9 s/4 s. The w/o emulsion formed was kept under constant stirring (1000 rpm) using a homogenizer (Ultra-turrax disperser, IKA Werke, Staufer, Germany) and simultaneously a 4% (w/v) solution of calcium chloride was sprayed over the emulsion at a constant flow rate of 0.75 mL/min to initiate interfacial ionic gelation of sodium alginate. The flow rate was controlled by connecting the sprayer to nitrogen source through a pressure controller valve. The spraying and stirring was continued until discrete and solid spheres were observed. The mixture containing suspended microspheres was cured by slowly adding 10 mL of aqueous solution of 8% (w/v) solution of $CaCl_2$ under constant stirring for 20 min at the same speed (1000 rpm) of sphere formation, followed by addition of 10 mL of isopropyl alcohol. Spheres were recovered from the liquid phase by filtering under vacuum. The recovered spheres were washed 2–3 times successively with copious amount of isopropyl alcohol to remove the oil phase from the spheres and the latter were dried under reduced pressure.

Preparation of SSD-Loaded Alginate Microspheres. SSD-loaded alginate microspheres were prepared by adding SSD to aqueous phase in various ratios (20, 25, 30, and 35% (w/w) of drug with respect to alginate) prior to sonication. The drug-loaded microspheres were developed by interfacial ionic gelation, followed by further rigidization according to the procedure mentioned earlier.

Process Variables and Their Effect on Microsphere Morphology and Size Distribution. The microsphere properties were optimized by analyzing the effect of the following process variables:

a. With different percentage of aqueous phase to oil phase for formation of w/o emulsion (core percentage with respect to oil phase include 10, 20, 30, 40, and 50% (v/v)).
b. By varying the sonication amplitude—30, 40, 50% (600 Hz).
c. By varying the stirring speed during ionic gelation process—700–1100 rpm range.

d. With different flow rate of spraying 4% (w/v) of $CaCl_2$ (in *n*-octanol)—0.5, 0.75, 1.0 mL/min.

The drug-loaded microspheres were analyzed for percentage SSD entrapment by subjecting a known amount of microsphere (100 mg) to concentrated nitric acid digestion and estimating the silver content using an atomic absorption (AA) spectrophotometer (Spectra varian AA Spectrophotomer, USA) in flame mode using an auto sampler. Background corrections were operated at a lamp current of 4 mA, slit width of 0.5 nm, and at a wavelength of 328.1 nm.

Further, the SSD loaded alginate microspheres were sized by Malvern diffraction particle size analyzer (Malvern Mastersizer-E Laser, UK). The particles were analyzed at a focal length of 328 mm by using isopropyl alcohol as a non-dissolving and non-reacting dispersion medium. The samples were kept at constant stirring using a magnetic cell stirrer till completion of analysis.

Preparation of SSD-Loaded Microsphere Impregnated Collagen Scaffold. Collagen was isolated from fetal calfskin after pepsin solubilization and salt precipitation according to Fuji and Khun.[36] The isolated collagen was extensively dialyzed against 0.05*M* acetic acid, lyophilized (Yamato-Neocool, Japan), and stored at 4°C.

For scaffold development, a known amount (300 mg) of pepsin-solubilized collagen was dissolved in 25 mL of 0.05*M* acetic acid. To the collagen solution a known amount (0.5 g) of drug-loaded microspheres was added and gently stirred to distribute the spheres homogeneously throughout the solution, and fibril formation was initiated by adding appropriate amount of 0.2*M* phosphate buffer (2 mL) and adjusting the pH to 6.9–7.2 using 2*N* sodium hydroxide, until turbidity appeared. After fibril initiation, the viscous solutions were uniformly cast over horizontally placed polypropylene platforms of $10 \times 10 \times 2$ cm^3 dimension. They were allowed to dry at a constant temperature of 34°C until a thin scaffold was obtained. The scaffold was then washed with distilled water twice or thrice to remove excess of salt and completely air-dried and stored in a light proof desiccator for further experimental purpose.

Determination of Distribution Homogeneity of the Microspheres in the Scaffold. To determine whether the drug-loaded microspheres are distributed in a homogeneous fashion, six equal contours (12.5 cm^2) were cut randomly from different drug impregnated scaffolds and were subjected for silver analysis by atomic absorption spectrophotometer and the amount of drug incorporated in each contour was determined.

Morphological Examination of Microspheres and Microspheres-Impregnated Collagen Scaffold. The surface morphology of the alginate microspheres, SSD loaded microspheres, and the SSD loaded alginate microspheres impregnated collagen scaffold were analyzed using JEOL 8401 scanning electron microscope, after spurt coating the samples with gold under vacuum.

To observe the morphology of the developed scaffold in simulated *in vivo* conditions, a 12.5 cm^2-scaffold was kept in a 60-mm culture Petri dish immersed in standard, Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) with 10% fetal bovine serum and observed till 48 h. The morphology of the collagen scaffold impregnated with microspheres was viewed under phase contrast microscope at 6, 12, 24, and 48 h and light microscopic pictures were taken.

***In Vitro* Release Studies.** A 12.5-cm^2 of microspheres-impregnated scaffold was placed over the aperture of Franz apparatus[37] and subjected to release rate experiment under constant stirring, using a magnetic stirrer, at 37°C. The reservoir solution of the Franz apparatus contained physiological synthetic serum electrolyte solution (SSES).[27] To determine the release characteristics, aliquots of samples were withdrawn at regular time intervals (6, 12, 24, 48, and 72 h) and analyzed for the amount of drug released using AA spectrophotometer.

***In Vitro* Antibacterial Efficiency Determination of Microspheres-Impregnated Collagen Scaffold.** Antibacterial activity of the drug-loaded spheres was examined against *Pseudomonas aeruginosa* (ATCC 25619), *Staphylococcus aureus* (ATCC 9144), *Klebsiella pneumoniae* (ATCC 15380), and *E. coli* (ATCC 25922) (IMTECH, Chandigarh, India). Initial inoculums of these strains were prepared by inoculating five colonies from fresh cultures (overnight cultured) in trypticase soy broth for *S. aureus, K. pneumoniae,* and *E. coli,* and in Mueller Hinton broth for *Pseudomonas aeruginosa*, incubated at 35°C till logarithmic growth phase. From this culture, 100 μL of the sample was transferred to 10 mL of respective media and incubated at 35°C to attain exponential logarithmic phase ($\equiv$ 0.5 McFarland). The cultures were appropriately diluted to get 5×10^5 cfu/mL and used as primary inoculum.[38,39] Different concentrations of SSD-loaded microspheres impregnated collagen scaffold were introduced into the flask containing various cultures and incubated at 35°C for 72 h. Samples were withdrawn at different time intervals to determine the minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) (99.9% kill from initial inoculums) and mean number of survivors at MBC.

RESULTS

Preparation of Alginate Microspheres

The process involves the use of *n*-octanol as a continuous phase to produce w/o emulsion. It is a low viscosity hydrophobic oil phase with higher polarity; therefore, to produce stable w/o emulsion, a surfactant with low HLB value (hydrophile lipophile balance value) is required, for which we have used 5% span 80 (HLB-4.5). Since homogeneous dis-

TABLE I. Influence of Process Variables on Morphology and Size Range of the Microspheres

S. No.	Variables Studied	General Aspects Observed	Analyzed Size Range (μm)
1	Core % ratio of alginate with respect to *n*-octanol + span 80 (v/v)		
	10	Small spheres, 80% aggregates, and undersized.	≤100–150
	20	More than 40% aggregates.	100–175
	30	Discrete spheres and 30% undersized.	200–300
	40	Discrete microspheres were obtained.	300–370
	50	Larger particles, oversized.	—
2	Sonication parameters[a] (% amplitude)		
	30	Large spheres mostly agglomerated.	>400
	40	Discrete and homogeneous sphere.	300–400
	50	Small spheres mostly undersized.	—
3	Stirring speed (opted during initiation of ionic gelation) (rpm)		
	800	Large irregular particles.	—
	900	40% spheres were recovered with large particles.	—
	1000	Discrete homogeneous spheres were recovered.	300–370
	1100	Discrete spheres, but with >40% aggregates.	≤200
	1200	Irregular, undersized aggregates of spheres.	≤200
4	Flow rate of $CaCl_2$ in *n*-octanol[b] (ml/min)		
	<0.5	Smaller, undersized spheres.	<150
	0.5	Discrete spheres were obtained but after longer time period (more than 45 min).	270–350
	0.75	Discrete and homogeneous spheres.	300–370
	≥1	Larger sphere along with aggregated particles.	—

[a] The effective sonication time period was kept constant at 15 min, since below this time discrete spheres were not obtained and resulted in aggregation and/or phase separation of emulsion.

[b] All the batch experiments were carried out using 4% (w/v) of $CaCl_2$ in *n*-octanol.

tribution of water phase in oil medium is determined by emulsification process, it is quite conventional to use high-speed homogenization to produce aqueous phase micelles of uniform size at the oil-surfactant medium. Because of the low viscosity of *n*-octanol, emulsification demands high-speed homogenization and takes a long time to produce a homogeneous w/o emulsion. To overcome this process, the emulsion has been prepared by sonication method, whereby the aqueous phase was added slowly to the oil phase and sonicated at an amplitude of 42% with 9-s pulse-on and 4-s pulse-off. It was previously observed[35] that at concentration above 5% (w/v) sodium alginate heterogeneous spheres are produced; hence, the experiments were carried out with concentrations below 5% (w/v) of sodium alginate. Since the primary emulsion process involves sonication, homogeneous emulsion was achieved with 2% (w/v) sodium alginate concentration.

The stable w/o primary emulsion, after sonication, was kept under constant stirring (1000 rpm) and polymerization was initiated by interfacial ionic gelation through 4 mL of $CaCl_2$ in *n*-octanol (4%, w/v). After microsphere collection, they were rigidized and thoroughly washed with isopropyl alcohol so that the oil phase is completely removed.

Effect of Process Variables on Microsphere Morphology

When the core percentage of aqueous phase to oil phase increased beyond 40% (v/v), agglomerated and heterogeneous spheres were obtained, while below 20% (v/v) ratio resulted in smaller spheres (<200 μm). From Table I it may be observed that 40% (v/v) produced spheres of expected size range and hence this concentration was used to prepare microspheres. Sonication for 9-s pulse-on with 4-s pulse-off for a total period of 15 min was effective and produced a homogeneous emulsion. By reducing the time period, the emulsion was found to be unstable during the subsequent processes. During the initiation of ionic gelation, homogenization speed beyond 1200 rpm resulted in aggregation while below 800 rpm resulted in heterogeneous agglomerated spheres. Speed

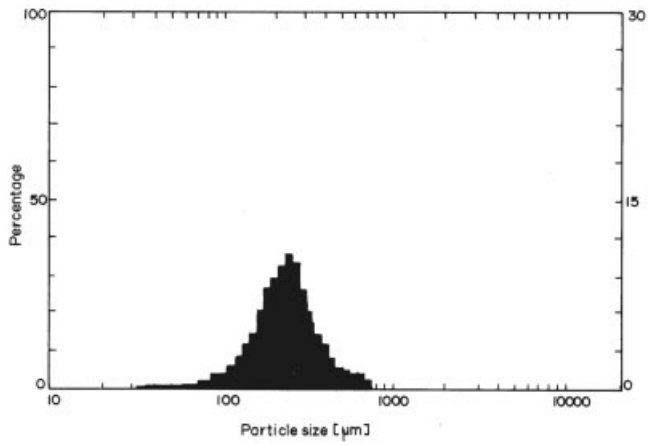

Figure 1. Particle size distribution of SSD-loaded microspheres: The size distribution pattern shows that more than 90% of spheres in the range of 300–364 μm.

range of 1000 rpm was observed to produce homogeneous spheres of desired range. Four-percentage (w/v) solution of $CaCl_2$ in *n*-octanol, upon spraying, enables slow and uniform cross-linking from the core to the surface. When added dropwise, however, immediate surface cross-linking with incomplete cores occurred, leading to increased surface aggregation, affecting the final collection of discrete microspheres. When the flow rate was increased (1 mL/min) surface aggregation occurred, while a flow rate below 0.5 mL/min resulted in increased time for sphere formation and the spheres formed were mostly undersized (<200 μm), hence the flow rate was maintained at 0.75 mL/min to produce homogeneous microspheres with uniform internal cross-linking without aggregation.

Influence of Process Variables on SSD-Percentage Entrapment and Size Distribution

With the aforementioned formulation parameter, the percentage drug loading was found to be 3.26%. From earlier studies it was obvious that the entrapment efficiency will be generally lower for low molecular weight compounds.[40] Though percentage entrapment was low, it was considered to be optimum when applied on wound site.[41] This was achieved by maintaining the initial drug load of 20% (w/w) with respect to alginate. Though the percentage could be increased, the formulation parameters were set to entrap the above-mentioned percentage of drug, so that when delivered, SSD released can be controlled within the therapeutic limit. The particle size ranges of microspheres were between 300 and 364 μm. As seen in Figure 1, more than 90% of spheres within the distribution curve are in the optimum size range.

Morphological Examination of Microspheres

Scanning electron microscopic images of alginate microspheres [Figures 2(a–c)] show their porous nature, and crevices observed indicate the diffusion controlled ionic gelation from the core. Higher flow rate (more than 1 mL/min of 4% (w/v) $CaCl_2$) caused aggregation and spheres tend to form a highly cross-linked rigid surface, whereas at a flow rate of 0.75 mL/min homogeneous spheres were obtained. During

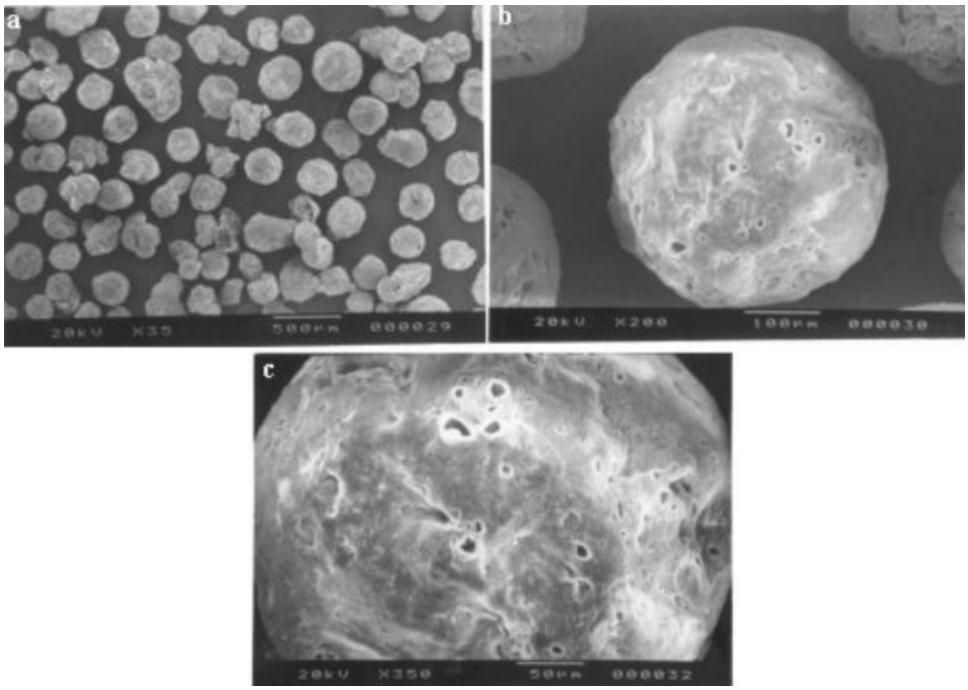

Figure 2. Scanning electron microscopic images of alginate microspheres: Porous nature of alginate microspheres under various magnifications shows the diffusion controlled ionic gelation from the core (magnification indicated in the images).

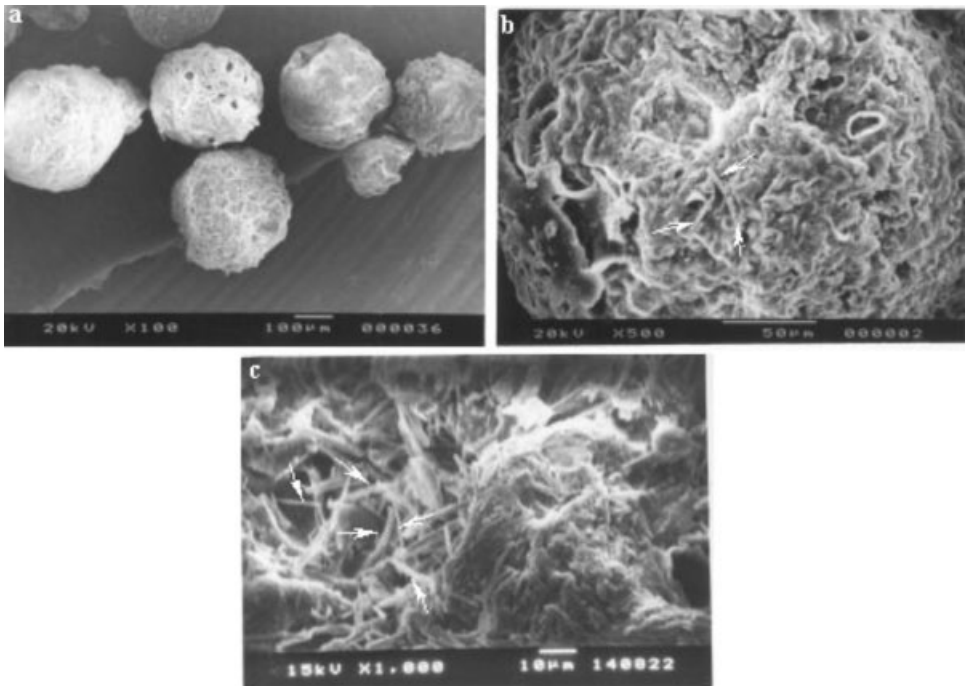

Figure 3. Scanning electron microscopic images of SSD-loaded alginate microspheres: SSD adhering in the interiors of the porous microspheres can be seen as needle-like crystals (indicated by the arrows) in (a), (b), and (c) (magnification indicated in the images).

SSD entrapment, the drug gets entrapped into the pores from inside, as it could be seen from SEM images [Figures 3(a–c)]. The needle-like structures (indicated by the arrow) show the crystals of SSD adhering to the pores of alginate microsphere, which prevents sticking by forming a physical barrier to the adjacent microspheres during the course of matrix congealing.

Morphological Features of SSD-Loaded Microsphere Impregnated Collagen Scaffold

Figure 4a shows the surface morphology of microsphere impregnated collagen scaffold, were the spheres are entangled in the fiber network. The interconnecting pores between fiber attachments to the microspheres can be seen in Figure 4(b). At high magnifications [Figure 4(c)] the overlaid microspheres entangled along with the collagen fibrils (as indicated by the arrow) can be observed. The SEM image [Figure 4(d)] of the section cut transverse to the surface of the collagen scaffold shows microspheres (as indicated by the arrow) impregnated within the porous interconnecting collagen fibril assembly.

Distribution Homogeneity Determination of SSD-Loaded Microsphere Impregnated in the Collagen Scaffold

When randomly cut, equally sized (12.5 cm^2) circular contours of known diameter of microsphere-impregnated scaffolds were subjected for silver analysis, they showed a great deal of homogeneous drug distribution pattern with negligible difference (Figure 5).

Assessment of Microspheres-Impregnated Collagen Scaffold Behavior *In Vitro*

The behavior of the microsphere-impregnated collagen scaffold in DMEM was assessed *in vitro* for 48 h. The light microscopic picture (20× magnification) taken immediately after immersing in DMEM shows the presence of microspheres entangled within the collagen scaffold [Figure 6(a)]. The release of microspheres [Figure 6(b)] from the scaffold surface (after 6 h) with drug present inside the matrix of microspheres indicates that SSD is protected from binding with protein backbone of the collagen, which otherwise would cause erratic drug release. The matrix morphology of alginate core at 12, 24, and 48 h can be observed in Figures 6(c–e) respectively, indicating that the drug is released as a function of time. In these pictures SSD appears in the form of black spots, which effectively decrease in number and intensity over time. At 24-h interval the entrapped drug at the peripheral regions are released completely, leaving drug at the core of the matrix. At 48 h very few SSD molecules could be seen and the matrix is almost left empty.

In Vitro Release Studies

The *in vitro* drug release experiments were carried using Franz diffusion apparatus. To simulate the *in vivo* ionic environment, SSES was used as a sink medium. From Figure 7 it can be observed that after 6 h around 47.5% of drug is released. This initial burst is a characteristic feature of an ionically cross-linked alginate microsphere[33] and is preferable since it is mandatory that any application intended to

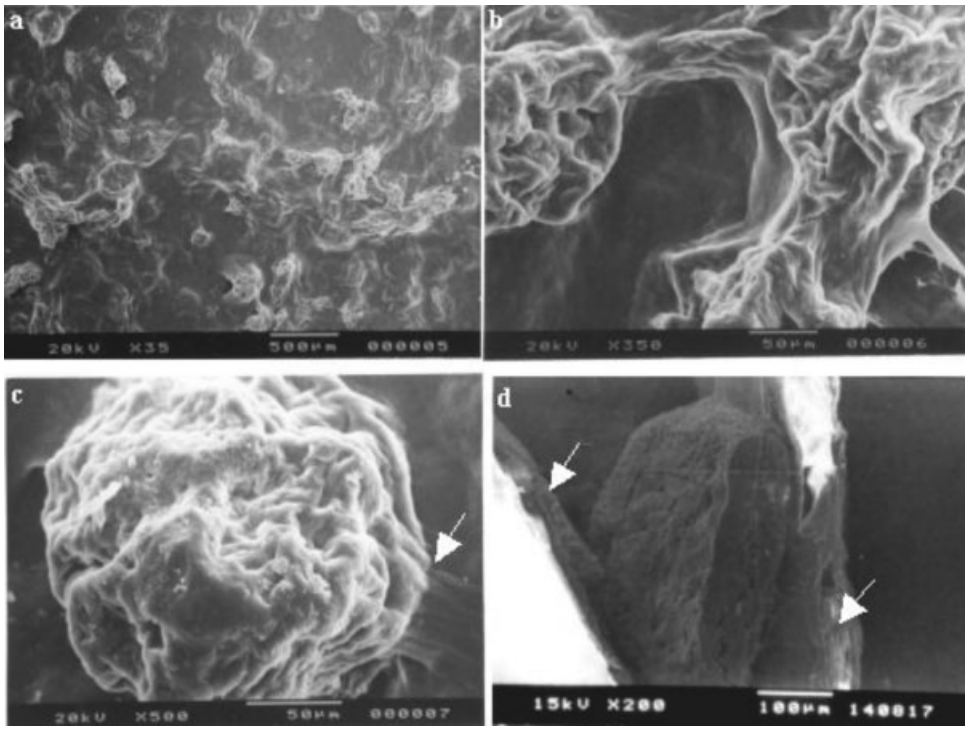

Figure 4. Scanning electron microscopic images of SSD loaded microspheres impregnated collagen scaffold: (a) The surface morphology of microspheres impregnated collagen scaffold, (b) The interconnecting pores between fiber attachments to the microspheres, (c) Overlaid microspheres entangled along with the collagen fibrils (indicated by arrow), and (d) The SEM image of section cut transverse to the surface of collagen scaffold (the arrow mark indicates the interconnecting collagen fibril). All magnifications indicated in the images.

control burn wound infection needs to exert immediate chemoprophylaxis. Subsequent analysis of released drug shows that around 53.4% drug was released at 12th h and 62.44%, 65.90%, and 68.8% of drug release were observed at 24-, 48-, and 72-h time intervals respectively. There was an observable increase in drug release between 12 and 24 h after which equilibrium concentration was achieved and maintained till 72 h with little increase in percentage release. Considering practical aspects, it is appreciable if the dressing was able to deliver SSD in a controlled fashion for a minimum of 3–4 days. This reduces the frequency of change of dressing in comparison to conventional cream-based therapy, which requires daily attention.

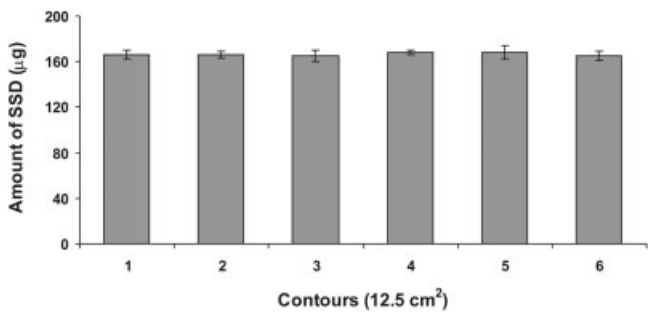

Figure 5. Distribution homogeneity determination in the collagen scaffold shows homogeneity with which SSD-loaded microspheres are distributed in the prepared collagen scaffold.

Antibacterial Efficiency Determination

Antibacterial efficiency of the SSD-loaded microspheres impregnated collagen scaffold was assessed by standard tube dilution method against four standard pathogenic strains (ATCC) to determine the MIC and MBC, using mid-logarithmic phase cultures. Equally sized scaffolds (12.5 cm^2) impregnated with various concentrations of microspheres were used in this experiment, of which, scaffold with 175 mg of microspheres (410 μg of SSD) was used for determining MBC using *Klebsiella pneumoniae* and *E. coli*, while scaffolds with 190 mg (450 μg of SSD) and 245 mg (580 μg of SSD) of microspheres were used against *Pseudomonas aeruginosa* and *Staphylococcus aureus* respectively. From Table II MIC was found to be 32 μg/mL for both *Klebsiella pneumoniae* and *E. coli*, while *Pseudomonas aeruginosa* and *Staphylococcus aureus* exhibited MIC levels of 44.8 and 57.6 μg/mL of SSD respectively. *Klebsiella pneumoniae* and *E. coli* exhibited survivors after 72 h (means of duplicate counts) at same MBC concentrations (40.2 μg/mL). Though the number of survivors observed varied, the concentrations at which they were susceptible are much lower for *Pseudomonas aeruginosa* (51.2 μg/mL) and *Staphylococcus aureus* (57.6 μg/mL).

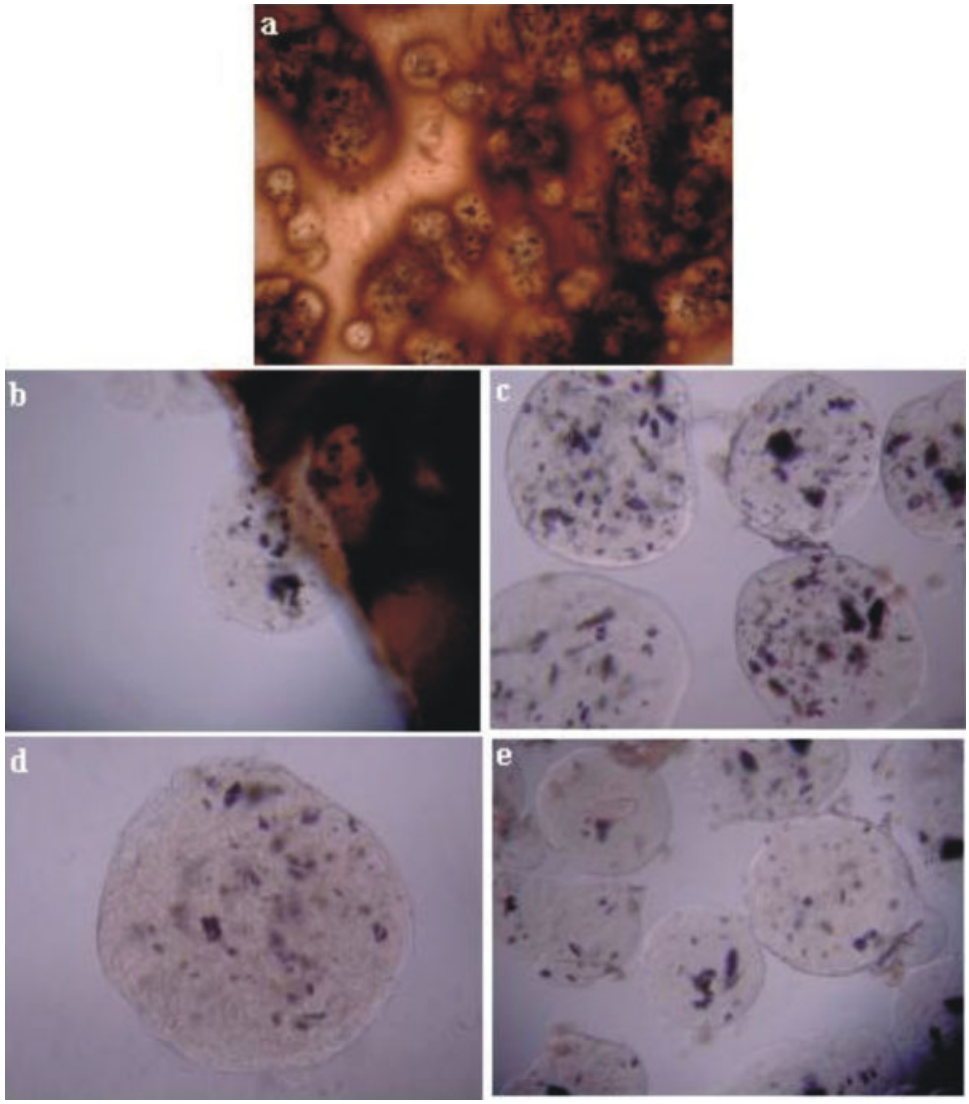

Figure 6. Light microscope pictures of microspheres-impregnated collagen scaffold behavior in DMEM: (a) shows the light microscopic picture taken immediately after immersing in DMEM. (b) shows the release of microspheres from the scaffold surface after 6 h. (c, d, and e) show matrix morphology of alginate core at 12, 24, and 48 h respectively (all pictures have been taken at 20× magnification). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

Alginate microspheres have been used extensively for delivering protein antigens,[33] but their ability as a microcarrier to deliver drug in a controlled manner at wound site has remained unexplored. The guluronic acid residues of sodium alginate preferentially bind calcium ions, producing cross-links capable of controlling the release rates of entrapped drug.[42] It is well known that at high concentrations of alginate, microspheres formed will have high internal and external cross-linking. From earlier investigations[40] it is known that low molecular weight compounds are entrapped at low concentrations. SSD used in the current study is a low molecular weight compound, which is a highly insoluble drug; therefore, its entrapment depends on the rate and internal

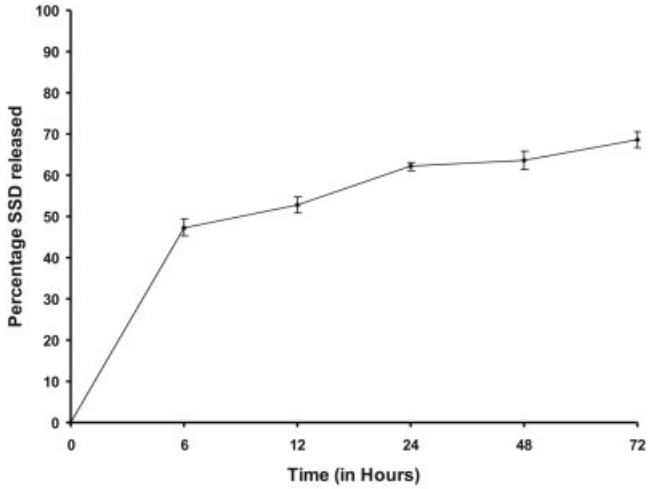

Figure 7. *In vitro* percentage SSD Release in SSES determined shows the controlled pattern of release for 72 h.

TABLE II. MIC and MBC of SSD-loaded Alginate Microspheres Impregnated Collagen Scaffold to Four Standard ATCC Reference Strains in Mid-Logarithmic Phase of Growth

Bacterial Strain	Concentration of SSD		Mean No. of Survivors After 3 $\log_{10}$ Reduction (MBC) after		
	MIC (μg/mL)	MBC (μg/mL)	24 h	48 h	72 h
E. coli	32	40.2	60	65	68
Klebsiella pnuemoniae	32	40.2	20	25	34
Pseudomonas aeruginosa	44.8	51.2	16	40	56
Staphylococcus aureus	57.6	57.6	7	16	24

polymerization of alginate. Higher degree of internal polymerization will impede the entrapment of SSD in the core of the microspheres. At 2% (w/v) concentration, the drug is entrapped even in the core, since cross-linking occurs from internal matrix to the surface during the movement of Ca^{2+} ions from oil–aqueous interface. As one of our main objectives is to deliver SSD at the site topically, so as to minimize the systemic internalization of silver ions, care was taken that SSD-loaded microspheres do not get engulfed into the peripheral blood vessels. Therefore the aforementioned process was used deliberately to produce microspheres of size range above 250 μm. This would obviate bolus entry of SSD-loaded microspheres and spheres would remain at the topical wound site.

Even though the topically applied dosage forms do not demand critical dosage levels, application of SSD over wound site is exceptional, because excessive exposure of drug, especially in cases of second degree partial thickness wound, might enable the drug to get absorbed through ruptured peripheral blood vessels. Earlier investigations provide evidence that silver binds to serum proteins and gets absorbed into systemic circulation.[27] Hence loading of 3.26% achieved by using 20% (w/w) of SSD with respect to alginate was just optimal so that after the initial burst release (~1.55%), therapeutic level of SSD could be attained easily and further maintained.[41] Though at higher concentrations of initial loading the drug can be entrapped at higher concentration, the process was optimized with 20% (w/w) concentration. At higher concentrations, SSD will get entrapped in the surface of the spheres without reaching the core; hence, it may result in bolus entry of the drug. Since delivery of SSD from alginate microspheres is through collagen scaffold, it is imperative that the entire wound surface is protected from external invasion of microbes. The aforementioned criterion was considered vital so that lower percentage of SSD entrapment would meet the necessary requirement. Hence low percentage loading (3.26%) would enable the homogeneous distribution of the microspheres over the entire wound surface; otherwise, the microspheres would be sparsely distributed. Morphological examination of the microspheres clearly showed that the porous surface of the microspheres has resulted because of cross-linking through a slow diffusion controlled mechanism. This enabled the crystalline SSD particles to reach the core and partially get attracted toward anionic sites of alginate, creating a weak interaction between polymer and drug, thus self-stabilizing the cores.

Impregnation of SSD-loaded microspheres into collagen was carried out *in situ* during collagen fibril formation to entangle the spheres between the fiber networks of collagen, which would cause interconnecting pores to increase in diameter when it swells. The obvious reason for initiating fibril formation in collagen is to minimize the interaction of collagen with spheres. This would actually prevent direct silver binding to collagen. If the drug was directly impregnated into the collagen scaffold, an erratic drug release will occur because of silver-protein binding. It is well known that during fibril formation inter cross-links of helical chains occur rather than the interaction of fibers with the microspheres; hence, release of SSD would purely depend on swelling of collagen and diffusion controlled release through alginate microspheres. Care was taken to produce a homogeneous distribution to maintain the drug concentration uniform throughout the wound surface.

The simulated *in vitro* conditions (both by DMEM and by SSES) exhibit the possible interaction of the released SSD from microspheres with the *in vivo* microenvironment. These models allowed the microspheres to hydrate slowly in a humid environment, as it would occur in the *in vivo* conditions. In the collagen scaffold, the embedded spheres subsequently swell, resulting in the release of drug and simultaneous ionization of SSD into silver and sulfadiazine moieties. During *in vitro* experiments, clear SSES solution in the reservoir compartment of Franz apparatus showed slight turbidity, indicating the ionization and subsequent association of silver ions with the chloride ions causing turbidity. This process of drug release is dependent on two aspects—first aspect is the porous interpenetrating network of collagen fibrils, which act as the primary rate limiting step and the second is the diffusion through porous alginate matrix. Depending on the level of hydration and equilibrium concentration of SSD, further release will occur.

The antibacterial efficiency of SSD-loaded microspheres impregnated collagen scaffold was determined by tube dilution method, since it was more appropriate than the agar diffusion method because of poor diffusivity of SSD through the agar surface.[43] It should be noted that the MIC levels

against *K. pnuemoniae, E. coli*, *P. aeruginosa,* and *S. aureus* were calculated with respect to the amount of total drug present in microspheres, which is expected to be released in a controlled fashion, and hence the MIC levels are on the higher side of the concentration range. The actual amount of released drug would be lesser and dependent on the rate of release and equilibrium concentration of SSD present at the wound site. Taking into account that dressing is intended to remain at wound site for at least three days, the experiment was designed and validated to determine the survivor count till 72 h.

The amount of microspheres impregnated in collagen scaffold was calculated in accordance with both the delivery rate and highest bacterial susceptibility concentration (with respect to *S. aureus* in our current investigation). All the experiments were performed using standard size of scaffold (12.5 cm^2). The actual amount of drug present in this particular area (165 μg) would justify the initial burst release rate ($\sim$1.55% $\equiv$ 78 μg) for attaining immediate chemoprophylaxis, equilibrium concentration maintenance in a controlled manner and antibacterial susceptibility. The above aspect and the homogeneous distribution to cover the entire area of wound surface during application were found to be crucial steps in designing the current system to deliver SSD in a controlled fashion for a period of 3 days, thus reducing the frequency of dressing.

In summary, currently developed collagen-based system has been designed to deliver SSD over the wound surface in a uniform and controlled fashion, bearing in mind the therapeutic dosage limit. The biomaterials used to develop this system are of natural origin, which lessens the worries of possible toxicity due to the core materials used. Apart from that, collagen and alginate by virtue possess active wound healing property. Collagen scaffold used as delivery device adds up more advantage to the present system, since it lessens dressing frequency, makes examination easier and assessment of wound site with added aesthetic value.

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