Volume II

New Frontiers in Biomaterials

Layne Burt

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Edited by **Layne Burt**

NY RESEARCH PRESS
New York

Published by NY Research Press,
23 West, 55th Street, Suite 816,
New York, NY 10019, USA
www.nyresearchpress.com

New Frontiers in Biomaterials: Volume II
Edited by Layne Burt

International Standard Book Number: 978-1-63238-345-7 (Hardback)

Printed in China.

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Preface

According to International Union of Physical and Applied Chemistry (IUPAC), biomaterials is defined as – material exploited in contact with living tissues, organisms, or microorganisms. It can be obtained either through natural processes or by synthesis in laboratories. They frequently find use as medical applications like synthetic heart valves and hip implants. In essence, they serve to replace the natural organ and serve the same function.

Additionally, they are also used in dentistry, surgery and as a medium to deliver various medical drugs. Occasionally, biomaterials are also grafted on to the body to assist in the delivery of drugs for a sustained period of time. Biomaterials also find extensive usage in the repair and replacement of skin in which cases new tissue is synthesised using the patient's skin itself. Another category of biomaterials is biopolymers, produced by living organisms. A perfect example of such polymer is cellulose.

Over the past few decades, rapid progress has been made in the field of biomaterials, given its rising demand and resultant technological breakthroughs. People have been working tirelessly behind the scenes to improve biomaterials and their efficacy. Countries like the Unites States have made giant strides of development in this branch of science.

This book is an effort to gather and organize these efforts and bring them to the readers in a comprehensive and lucid manner. I would like to thank all those who have contributed to this book at any step. Your efforts are deeply appreciated. I would like to particularly thank my family for their endless support and faith in me.

Editor

Contents

The Effect of Zirconia in Hydroxyapatite on *Staphylococcus epidermidis* Growth

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Academic Editor: Jukka Pekka Matinlinna

Synthetic hydroxyapatite (HA) has been widely used and developed as the material for bone substitute in medical applications. The addition of zirconia is needed to improve the strength of hydroxyapatite as the bone substitute. One of the drawbacks in the use of biomedical materials is the occurrence of biomaterial-centred infections. The recent method of limiting the presence of microorganism on biomaterials is by providing biomaterial-bound metal-containing compositions. In this case, *S. epidermidis* is the most common infectious organism in biomedical-centred infection. *Objective.* This study was designed to evaluate the effect of zirconia concentrations in hydroxyapatite on the growth of *S. epidermidis*. *Methods and Materials.* The subjects of this study were twenty hydroxyapatite discs, divided into four groups in which one was the control and the other three were the treatment groups. Zirconia powder with the concentrations of 20%, 30%, and 40% was added into the three different treatment groups. Scanning electron microscope analysis was performed according to the hydroxyapatite and hydroxyapatite-zirconia specimens. All discs were immersed into *S. epidermidis* culture for 24 hours and later on they were soaked into a medium of PBS. The cultured medium was spread on mannitol salt agar. After incubation for 24 hours at 37°C , the number of colonies was measured with colony counter. Data obtained were analyzed using the ANOVA followed by the pairwise comparison. *Result.* The statistical analysis showed that different concentrations of zirconia powder significantly influenced the number of *S. epidermidis* colony ($P < 0.05$) . *Conclusion.* The addition of zirconia into hydroxyapatite affected the growth of *S. epidermidis*. Hydroxyapatite with 20% zirconia proved to be an effective concentration to inhibit the growth of *S. epidermidis* colony.

1. Introduction

Angiogenesis, osteogenesis, and chronic wound healing are natural repairing mechanisms that occur in human body. However, there are some critical defects of size in which these tissues cannot regenerate themselves and need clinical repair [1]. Therefore, the treatment for posttraumatic skeletal conditions such as bone loss is becoming a challenging field to be studied [2]. In most cases, restoration of alignment and stable fixation of the bone is necessary to achieve a successful reconstruction. Bone grafts have an important role in orthopaedic surgery, as well as in the replacement of bone after a trauma or tumour removal [3]. In many cases, adjunctive measures such as bone grafting or bone transports are required to stimulate bone healing and fill the bone defects [2]. Autologous cancellous bone is the most effective biological graft material [4]. It is the most preferable procedure for bone augmentation because of osteoinductive effect and high biocompatibility. However, limitation of available amount and postoperative discomforts including inflammation at donor site are the disadvantages [5]. Skeletal bones comprise mainly of collagen and hydroxyapatite, both are osteoconductive components [1]. Nowadays hydroxyapatite, $[Ca_{10}(PO_4)_6(OH)_2]$, as a very important bioceramic is used extensively in medical applications to repair or replace the bone tissues [6, 7]. Dentistry and orthopaedic applications deal a lot with this material because of its good biocompatibility, osteoconductivity, and the bone-bonding

properties [8]. Pure hydroxyapatite has chemical composition, biological and crystallographic properties which are highly similar to the bone and the teeth [9].

Hydroxyapatite (HA) powder can be synthesized from various minerals, including coral, gypsum ($CaSO_4 \cdot 2H_2O$), and calcite [1]. Some methods have been developed in preparing HA powders, the wet methods and solid state reactions. The wet methods can be divided into hydrolysis and hydrothermal techniques. The hydrothermal technique provides HA powder with high degree of crystallinity with Ca-P ratio which is close to the stoichiometric value [10]. It is proved that HA powder can be synthesized from local gypsum using microwave-hydrothermal method [11].

Bulk hydroxyapatite exhibits poor mechanical properties, therefore, for full utilization that must withstand high loads, the improvement of mechanical properties is needed [6]. The primary purpose of adding filler particles is to strengthen a composite [12]. Zirconia is one of the important filler among many fillers or reinforcements, which has been used to increase the strength and toughness of many ceramic materials [7]. In bone surgery zirconia has shown very wide applications [13].

One of the major drawbacks in the use of biomaterials is the occurrence of biomaterial-centred infections [14]. After implantation, the host will interact with biomaterial by forming a conditioning film on its surface. Adherence of microorganisms is mediated by the properties of the biomaterial surface itself. Subsequent surface growth of microorganism will initiate the occurrence of infection. An *in vitro* study proved that the number of microorganism *S. epidermidis* increased in the first 8 to 12 hours after the implantation [14]. Efforts have been done in preventing the contamination of microorganism on the foreign materials during implantation. No doubt that the use of antibiotics might reduce the occurrence of biomaterials-centred infection, but still a significant number of patients are suffering from this condition. The aim of this research was to examine the effect of zirconia on the growth of *S. epidermidis*.

2. Materials and Methods

2.1. Synthesis of Local Hydroxyapatite and Discs Preparation. The synthesis of hydroxyapatite was conducted and modified [15] to produce powder of hydroxyapatite from local gypsum (Kulon Progo Yogyakarta, Indonesia). The gypsum powder was obtained by pulverizing the gypsum rock. Gypsum powder (20 gr) and 800 mL of 1 M diammonium hydrogen phosphate [$(NH_4)2HPO_4$] were well mixed and treated at 100°C for 20 minutes in a pyrex glass using a microwave digestion system. The system was operated at frequency of 2.45 GHz. After the hydrothermal reaction, reacted sample was washed with distilled water to remove residual ion and dried. The conversion of gypsum to L-HA was estimated from the ratio of X-ray intensities of gypsum peak (d = 7.261) and the HA peak (d = 2.787) using powder X-ray diffractometry.

Powder of hydroxyapatite (0.4 gr) was put in a mould of compaction instrument [11]. Powder of hydroxyapatite was mixed homogenously with zirconia. In this study the different concentrations of zirconia used were 10%, 20%, 30%, and 40% (weight/weight). Powder of hydroxyapatite-zirconia is put in a mould and pressed with 120 Mpa to produce a disc with a diameter of 10 mm and 3 mm in thickness. Finally, discs were sintered for 2 hours at 1450°C. The sterilization of the hydroxyapatite-zirconia discs was done by keeping the discs in the autoclave for 15 minutes at 121°C. Twenty discs were prepared and divided into five discs for each group of concentration.

2.2. Scanning Electron Microscope Examination. The disc specimens were mounted with silver paste on metallic stub. The specimens were then gold coated with a sputtering system under vacuum desiccation. Examination was performed under scanning electron microscope at an acceleration voltage of 7 to 10 KV.

2.3. S. epidermidis Culturing and Colonies Counting. The *S. epidermidis* (clinical strain) was cultured in mannitol salt agar (MSA) and incubated for 24 hours at 37°C. The cultured bacteria was then transferred into 2 mL of brain heart infusion (BHI) and incubated for 24 hours at 37°C. Solution of NaCl was added into the BHI. Five discs of hydroxyapatite-zirconia from each group of concentration were put into 3 mL culture of *S. epidermidis* in BHI (Brown standard III of 10^8 CFU/mL). Incubation was followed for 24 hours at 37°C. After incubation, all discs were transferred into 1 mL phosphate buffer saline (PBS) and put on a vibrator to remove the attached *S. epidermidis*. Without taking measurement, all discs were then taken out from the PBS solution, and the solution was diluted up to 10^{-4}. Next step, 0.1 mL medium of BHI was transferred into the MSA at the petri dish, using a spreader. The petri dish was incubated for 24 hours at 37°C. The number of colonies was counted using colony counter.

3. Discussion

The average and standard deviations of the *S. epidermidis* number of colony on MSA when reacted with hydroxyapatite mixed with zirconia at concentrations of 20%, 30%, and 40% are presented at Table 1.

On Table 1 it is noted that the colony number of *S. epidermidis* is consistently influenced by zirconia concentrations in the hydroxyapatite. It is shown that the number of colonies of *S. epidermidis* was smaller as the concentration of zirconia in the hydroxyapatite was increased. In the group of hydroxyapatite without zirconia, it shows the most number of colonies, whereas the concentration of 40% shows the smallest number of colonies. Statistical analysis of this data is carried out using the one-way analysis of variance. This analysis proved whether the different concentrations of zirconia have significant influence on the growth of *S. epidermidis*, and it is indicated by the numbers of the colonies of *S. epidermidis*. The probability from the ANOVA is 0.001, and this value is less then 0.05 confidence levels. From this result it was proved that hydroxyapatite with different concentrations of zirconia had a significant influence on the number of colonies of *S. epidermidis*.

Table 1: Measurement of the number of colony of *S. epidermidis* after being reacted with hydroxyapatite mixed with different concentrations of zirconia.

Replications	Concentrations of zirconia			
	0%	20%	30%	40%
1	30	15	7	3
2	57	16	8	0
3	64	14	10	0
4	32	16	7	0
5	25	12	12	0
x	**41.6**	**14.6**	**8.8**	**0.6**
$x \pm$ sd	**41.6 ± 17.61**	**14.6 ± 1.67**	**8.8 ± 2.16**	**0.6 ± 1.34**

Table 2: Statistical result of the pairwise comparisons from hydroxyapatite with different concentrations of zirconia on the number of colonies of *S. epidermidis*.

(*I*) VAR0000	(*J*) VAR0000	Mean difference (*I*-*J*)	Std. error	Sig.	95% Confidence interval	
1	1				Lower bound	Upper bound
1.0	2.00	27.00000*	5.65332	.000	15.0155	38.9845
	3.00	32.80000*	5.65332	.000	20.8155	44.7845
	4.00	41.00000*	5.65332	.000	29.0155	2.9845
2.0	1.00	−27.00000*	5.65332	.000	−38.9845	−15.0155
	3.00	5.80000	5.65332	.320	−6.1845	17.7845
	4.00	14.00000*	5.65332	.025	2.0155	25.9845
3.0	1.00	−32.80000*	5.65332	.000	−44.7845	−20.8155
	2.00	−5.8000	5.65332	.320	−17.7845	6.1845
	4.00	8.20000	5.65332	.166	−3.7845	20.1845
4.0	1.00	−41.00000*	5.65332	.000	−52.9845	−29.0155
	2.00	−14.00000*	5.65332	.025	−25.9845	−2.0155
	3.00	−8.20000	5.65332	.166	−20.1845	3.7845

* The mean difference is significant at the 0.05 level.

The result of this study coincided with the research which stated that the factors influencing bacteria adherence to a biomaterial surface include the surface roughness or physical configuration of the material [16]. The microstructure of hydroxyapatite and hydroxyapatite-zirconia had been tested using scanning electron microscope, and the result proves that hydroxyapatite zirconia has smoother surface roughness than hydroxyapatite [13]. Furthermore, it is stated that the particles of zirconia fill in the pores among the particles of hydroxyapatite [17]. This statement coincides with the scanning electron microscope examination as presented in Figures 1 and 2.

It can be understood that hydroxyapatite-zirconia has less pores compared to hydroxyapatite without zirconia. Material with least pores among particles gives smoother surface of the material. The result of the ANOVA proved that there was significant relation between the zirconia concentrations and the colonies growth of *S. epidermidis* ($P < 0.05$). The number of colonies in hydroxyapatite-zirconia was influenced by the addition of zirconia. This situation related to the statement which mentioned that the present invention provides a method of limiting the presence of a microorganism by contacting the microorganism with material-bound metal-containing compositions [18]. One of the commonly used filler which contain heavy metal is zirconia [12]. The small number of colonies of *S. epidermidis* in hydroxyapatite-zirconia might be assumed due to the toxicity of zirconia as metal. ANOVA analysis was followed by the pairwise comparisons.

The data in Table 2 show significant differences in almost all pair of groups, except the differences between (20–30)% and (30–40)%. Based on this result it can be drawn that zirconia with the concentration of 20% proved to have been an effective concentration in inhibiting the growth of *S. epidermidis*. Moreover, as 20% becomes the effective concentration, a detailed research is still needed. It is important to find out the most effective concentration of zirconia to be added into hydroxyapatite.

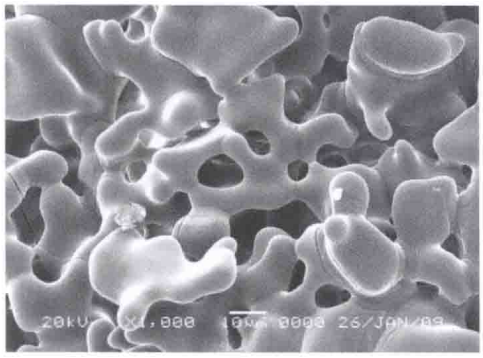

FIGURE 1: Microstructure of hydroxyapatite.

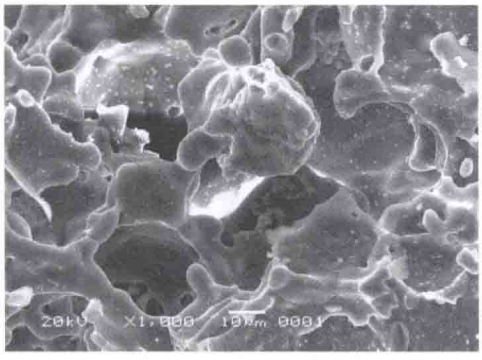

FIGURE 2: Microstructure of hydroxyapatite with zirconia fill in the pores.

4. Conclusions

From this study, it can be concluded that concentration of zirconia influenced the number of colonies of *S. epidermidis*. Local hydroxyapatite with 20% zirconia proved to have been an effective concentration in inhibiting the growth of *S. epidermidis*. Scanning electron microscopy examination showed that zirconia filled in the hydroyapatite pores, whereas the less pores the less number of *S. epidermidis* attached.

Acknowledgment

This paper has been presented at the 88th IADR General Session and Exhibition, held at Barcelona, Spain, on July 14–17, 2010.

References

[1] D. A. Wahl and J. T. Czernuszka, "Collagen-hydroxyapatite composites for hard tissue repair," *European Cells and Materials*, vol. 11, pp. 43–56, 2006.

[2] C. G. Finkemeier, "Bone-grafting and bone-graft substitutes," *Journal of Bone and Joint Surgery A*, vol. 84, no. 3, pp. 454–464, 2002.

[3] Y. H. Hsu, I. G. Turner, and A. W. Miles, "Fabrication of porous bioceramics with porosity gradients similar to the bimodal structure of cortical and cancellous bone," *Journal of Materials Science*, vol. 18, no. 12, pp. 2251–2256, 2007.

[4] S. Bansal, V. Chauhan, S. Sharma, R. Maheshwari, A. Juyal, and S. Raghuvanshi, "Evaluation of hydroxyapatite and beta-tricalcium phosphate mixed with bone marrow aspirate as a bone graft substitute for posterolateral spinal fusion," *Indian Journal of Orthopaedics*, vol. 43, no. 3, pp. 234–239, 2009.

[5] K. Tint, H. Kondo, S. Kuroda et al., "Effectiveness of extracted teeth as bone substitute, application to parietal bone defects in rabbit," *Journal of Oral Tissue Engineering*, vol. 3, no. 1, pp. 7–16, 2005.

[6] Q. Wang, S. Ge, and D. Zhang, "Highly bioactive nano-hydroxyapatite partially stabilized zirconia ceramics," *Journal of Bionics Engineering*, vol. 1, no. 4, pp. 215–220, 2004.

[7] Y. Nayak, R. P. Rana, S. K. Pratihar, and S. Bhattacharyya, "Pressureless sintering of dense hydroxyapatite-zirconia composites," *Journal of Materials Science*, vol. 19, no. 6, pp. 2437–2444, 2008.

[8] C. G. Simon, J. M. Antonucci, D. W. Liu, and D. Skrtic, "In vitro cytotoxicity of amorphous calcium phosphate composites," *Journal of Bioactive and Compatible Polymers*, vol. 20, no. 3, pp. 279–295, 2005.

[9] M. K. Herliansyah, M. Hamdi, A. I. Ektessabi, and M. W. Wildan MW, "Fabrication of hydroxyapatite bone graft for implant applicationa literature study," in *Proceedings of the First International Conference on Manufacturing and Material Processing*, pp. 559–564, Kuala Lumpur, Malaysia, 2006.

[10] W. Suchanek and M. Yoshimura, "Processing and properties of hydroxyapatite-based biomaterials for use as hard tissue replacement implants," *Journal of Materials Research*, vol. 13, no. 1, pp. 94–117, 1998.

[11] E. E. Pujiyanto, A. E. Tontowi, M. W. Wildan, and W. Siswomihardjo, "Sintesis hidro-Hidroksiapatit dari gipsum Tasikmalaya sebagai bahan baku produk tulang buatan," in *Seminar on Aplication and Research in Industrial Technology*, pp. 119–126, Jur Teknik Mesin dan Industri UGM, Yogyakarta, Indonesia, 2006.

[12] K. J. Anusavice, *Phillip's Science of Dental Materials*, Elsevier, 11th edition, 2009.

[13] R. Quan, D. Yang, X. Wu, H. Wang, X. Miao, and W. Li, "In vitro and in vivo biocompatibility of graded hydroxyapatite-zirconia composite bioceramic," *Journal of Materials Science*, vol. 19, no. 1, pp. 183–187, 2008.

[14] B. Gottonboss, *The development of antimicrobial biomaterial surface [thesis]*, Rijks University of Groningen, 2001.

[15] H. Katsuki, S. Furuta, and S. Komarneni, "Microwave versus conventional-hydrothermal synthesis of hydroxyapatite crystals from gypsum," *Journal of the American Ceramic Society*, vol. 82, no. 8, pp. 2257–2259, 1999.

[16] M. Katsikogianni and Y. F. Missirlis, "Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions," *European Cells and Materials*, vol. 8, pp. 37–57, 2004.

[17] E. S. Ahn, N. J. Gleason, and J. Y. Ying, "The effect of zirconia reinforcing agents on the microstructure and mechanical properties of hydroxyapatite-based nanocomposites," *Journal of the American Ceramic Society*, vol. 88, no. 12, pp. 3374–3379, 2005.

[18] K. D. Landgrebe, D. J. Hastings, T. P. Smith, G. D. Cuny, A. Sengupta, and C. D. Brandys, Limiting the presence of microorganisms using polymer-bound metal-containing compositions, 2010, http://www.freepatentsonline.com/6432396.html.

Encapsulation of Liposomes within pH Responsive Microspheres for Oral Colonic Drug Delivery

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Academic Editor: Xian Zheng Zhang

A novel liposome-in-microsphere (LIM) formulation has been created comprising drug-loaded liposomes within pH responsive Eudragit S100 microspheres. The liposomes contained the model drug 5-ASA and were coated with chitosan in order to protect them during encapsulation within the microspheres and to improve site-specific release characteristics. *In vitro* drug release studies showed that LIMs prevented drug release within simulated stomach and small intestine conditions with subsequent drug release occurring in large intestine conditions. The formulation therefore has potential for oral colonic drug delivery.

1. Introduction

Within oral drug delivery, specific targeting of the colon can be advantageous due to near-neutral pH, low enzyme and bile salt activity and long residence time. For local treatment of colonic diseases, direct targeting may increase drug bioavailability at the target site, therefore allowing reductions in administered dose and systemic side effects [1]. It has also been shown that specific targeting to the colon is advantageous for systemic treatments for a number of reasons including the potential for protein and peptide drug absorption [1, 2]. However, oral drug delivery to the colon is associated with a number of obstacles including dosage form transit through regions of high acidity and digestive activity.

Liposomes are drug carriers that can be used for a wide range of active ingredients [3], have the ability to interact with cells [4], and have potential in gene transfection [5]. Evidence also suggests that they can advantageously interact with colonic tissue and/or find utility in colonic drug delivery [6–9]. However there is little information on how they could be delivered to this region, particularly via the oral route, which is generally the favoured route for drug administration. Liposomes are not naturally suited to oral drug delivery due to their susceptibility to digestion en route through the GI tract. Coating them with a polymer is one way that may protect them during transit, but very little work has been done on specifically targeting the colonic region.

Previously, we have described the direct coating of liposomes with the methacrylic acid copolymer Eudragit S100, as a means to facilitate colonic targeting following oral administration [10]. With its anionic carboxylic acid side groups and solubility threshold of pH 7, the Eudragit S100 coat allowed an appropriate pH-dependent drug release profile to be achieved, but the coat was not able to prevent ingress of bile salts, which would lead to premature drug release *in vivo*. The aim of the current work was therefore to improve that formulation and to create a novel liposome-in-microsphere (LIM) system comprising chitosan-coated liposomes surrounded by a solid shell of Eudragit S100. Chitosan, as a polymer resistant to the organic solvents used in microsphere production, was included to protect the liposomes during their encapsulation. Chitosan is also known to be solubilised by the specific polysaccharidases (glucosidases, glycosidases), which are secreted by colonic bacteria, through the random scission of the 1,4 glycosidic bond [11, 12]. Therefore its incorporation would also facilitate colonic targeting via a mechanism discrete to that imparted by the Eudragit S100. Given that inter- and intrapatient variation

in gastrointestinal (GI) tract conditions (particularly pH) can be significant, formulations reliant on more than one physiological trigger may provide a more accurate means of delivery to the colon [13].

2. Materials and Methods

2.1. Materials. Liposomal membrane components included egg phosphatidylcholine (EPC) (a gift from Lipoid, Ludwigshafen, Germany, minimum 98% purity), cholesterol (CH) (Sigma Aldrich, Dorset, UK), and dicetyl phosphate (DCP) (Sigma Aldrich). 5-aminosalicylic acid (5-ASA)(Sigma Aldrich) was chosen as it is an anti-inflammatory drug used in the treatment of ulcerative colitis and Crohn's disease. Chitosan (low molecular weight measured at 237,000 by gel permeation chromatography, Sigma Aldrich) was used to coat the liposomes. Eudragit S100, the pH responsive polymer used for producing the microspheres, was a gift from Evonik (Essen, Germany). For the drug release studies 0.1 M hydrochloric acid (HCl), Hanks' balanced salt solution (99.015 mol% water, 0.95% Hanks' balanced salt and 0.035% sodium bicarbonate adjusted to pH 6.3 using 0.1 M HCl), and phosphate buffered saline (PBS, pH 7.4) were used to simulate the pH conditions of the stomach [12, 14], small intestine [14], and colonic region, respectively. All components for the release media were purchased from Sigma Aldrich (Dorset, UK). Sodium taurocholate (ST) (10 mM) was used as a model bile salt in the small intestine buffer [15] and β-glucosidase (4% w/v, $\geq$ 24,000 units/100 mL) from almond emulsin (Sigma Aldrich) was added to the PBS as its chitinase activity is considered to be representative of that occurring in the colonic region [16–18]. All other chemicals and solvents used were purchased from Fisher Scientific and used as received.

2.2. Preparation Methods

2.2.1. Formulation of Liposomes and Subsequent Coating with Chitosan. Liposomes were prepared using EPC and CH in the molar ratio 7 : 2, with DCP comprising 10% of the total lipid for anionic formulations. The conventional thin film hydration method [19] was used to produce multilamellar vesicles (MLVs), which were then extruded to produce large unilamellar vesicles (LUVs) for the study. Briefly, the lipids were dissolved in 5 mL chloroform in a 50 ml round bottom flask. The chloroform was then removed using a rotary evaporator, leaving a thin lipid film on the side of the flask which was then dried under nitrogen for 2 hours to remove trace chloroform. The film was then hydrated with an aqueous solution containing 1 mg/ml 5-ASA in PBS (pH 7.4). During hydration the flask was agitated using a vortex mixer. Extrusion was carried out using an Avanti Lipid miniextruder through membranes with progressively smaller pores (1 μm, 0.4 μm and 0.2 μm). Each sample was passed through each membrane fifteen times, producing vesicles with a narrow size distribution. Excess drug was removed through three cycles of centrifugation (63,000 relative centrifugal force (rcf)) and replacement of supernatant with PBS. The final pellet was then resuspended in 10 mL of PBS.

To prepare the coated liposomes equal volumes of liposomal suspension and aqueous solution of chitosan of various concentrations (0.25, 0.5, 1, 2 and 3% w/v in 1% acetic acid) were combined. Liposomal suspensions were added dropwise to the chitosan solution whilst under magnetic stirring, with the stirring being continued for a further 5 minutes [20–22]. The chitosan-coated liposomes were then left at 4°C for 24 hours to allow them to stabilise [23–25]. Excess chitosan was then removed by washing three times by centrifugation (63,000 rcf) and replacement of supernatant with 1% acetic acid.

2.2.2. Encapsulation of Chitosan-Coated Liposomes within Eudragit S100 Microspheres. Chitosan-coated LUVs were encapsulated within Eudragit S100 microspheres using a double emulsion-solvent evaporation technique developed from previous work by Park et al. [26]. Eudragit S100 was dissolved in a solvent mixture of DCM:ethanol:propanol (5 : 6 : 4) to produce a 6% solution (w/w). 5 mL of the organic solution was added to a water phase comprising 0.8 mL of the chitosan-coated LUV suspension and 0.2 mL polysorbate 20 (3% w/w) which had previously been vortex mixed (2,400 rpm, Fisherbrand FB15024). The primary emulsion [W_1/O] was then formed by homogenising the solution for 2 minutes at 7,400 rpm (IKAT25 homogeniser, Fisher Scientific). The primary emulsion was then poured into 100 mL 1% PVA whilst under magnetic stirring at 125 rpm, thus creating the double emulsion [W_1/O/W_2]. The LIMs were magnetically stirred for 3 hours for subsequent polymer hardening and solvent evaporation. LIMs were then harvested by washing and vacuum filtration (filter membrane 1.6 μm) with 200 mL distilled water to remove any excess surfactant.

2.3. Characterisation of Formulations

2.3.1. Zeta Potential. Changes in dispersion zeta potential as a function of chitosan concentration were determined through electrophoretic mobility measurements (Zetamaster, Malvern Instruments, UK). Briefly, 500 μL of the liposome/polymer suspensions (from Section 2.2.1) were diluted with 20 mL of distilled water (pH < 7) before introducing to the electrophoresis cell. Ten measurements were taken at 25°C on three samples from three independent formulations.

2.3.2. Size Distribution. Vesicle size and size distribution before and after coating with chitosan were measured using wet laser diffraction particle sizing (Mastersizer 2000 connected to a Hydro SM small volume sample dispersion unit, Malvern Instruments, UK). Measurements were carried out in distilled water in which the polymer was not soluble. Three independent formulations of each preparation were each measured 5 times.

2.3.3. FITC Labelling of Chitosan and Subsequent Fluorescence Microscopy. To visualise the chitosan coating layer on the liposomes a method used by Amin et al. [24] was