

BIODEGRADABLE MICROSPHERES WITH ENHANCED
CAPACITY FOR SURFACE LIGAND CONJUGATION

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Mark Edwin Keegan

May 2004

© 2004 Mark Edwin Keegan

BIODEGRADABLE MICROSPHERES WITH ENHANCED CAPACITY FOR SURFACE LIGAND CONJUGATION

Mark Edwin Keegan, Ph.D.

Cornell University 2004

A poly(lactic-co-glycolic acid) (PLGA) microsphere formulation was developed which incorporates carboxylic acid groups into the microsphere surface. These functional groups are suitable for coupling to a variety of ligands, and form linkages that remain stable in aqueous environments for extended periods of time. The ligand binding capacity of these microspheres compares favorably to that of comparably sized carboxylated microspheres, which are commonly used as model particles for targeted microsphere delivery studies. The morphology and drug release kinetics of this PLGA microsphere formulation are not significantly different from those of microspheres made with traditional reagents. A variety of different protein ligands can be conjugated to the surfaces of these microspheres. These microsphere-ligand conjugates were then used in model systems to evaluate the effect of conjugated ligands on microsphere behavior. Microsphere retention in agarose columns was increased by ligands on the microsphere surface specific for receptors on the agarose matrix. In another experiment, conjugating the ligand *Ulex europaeus* agglutinin 1 to the microsphere surface increased the adhesion of microspheres to Caco-2 monolayers compared to control microspheres. This increase in microsphere adhesion was negated by co-administration of L-fucose, indicating that the increase in adhesion is due to specific interaction of the ligand with carbohydrate receptors on the cell surface. These results demonstrate that the ligands conjugated to the microspheres maintain their receptor binding activity, and are present on the microsphere surface at

a density sufficient to target the microspheres to both monolayers and three-dimensional matrices bearing complimentary receptors. These microspheres combine the capability to target specific cell types through surface-conjugated receptors with the ability to release encapsulated drugs over extended periods of time. This combination of properties enhances the utility of biodegradable microspheres for a variety of drug delivery applications.

BIOGRAPHICAL SKETCH

Mark Keegan was born on a Monday morning in February in Burlington, Vermont. As a young student, he discovered both a passion and an aptitude for making things bubble, spark, and catch fire, which on occasion would be displayed within the structured confines of science class. Not one to fight an obvious calling, he majored in chemical engineering at Northeastern University, and minored in late-night card games with his roommates. While a freshman at Northeastern, he met a girl named Clara Holt. The two of them bubbled, sparked, and eventually caught fire. Mark spent the next 8 years working up the nerve to ask her to marry him. In his spare time, he entered the Ph.D. program in chemical engineering at Cornell University, and in the time between pickup hockey games he managed to produce the dissertation you are now reading. In the future he hopes to complete life's circle by returning to Vermont to introduce young students to some of the wonders of science – especially things that bubble, spark, and catch fire.

For those who, if forced to decide between doing well and doing good, would choose the latter.

ACKNOWLEDGMENTS

First, I would like to thank my advisor, Mark Saltzman. Thinking back on our discussions over the course of this project, I don't recall ever hearing the word "no" in response to any of the ideas I proposed to pursue. I'm certain that that's not because all my ideas were good ones, but because Mark makes a dedicated effort to allow his students to follow their own paths as much as possible. I've enjoyed a level of independence that not all graduate students have in the pursuit of their dissertations, and I am grateful. I'd also like to thank my other committee members, Mike Shuler and Judy Appleton. Mike was particularly helpful with regards to the cell culture experiments described in Chapter 3, which was trickier than that chapter lets on. Judy was the first person to suggest to me that flow cytometry might be useful in my research, and readers able to persevere through the length of this dissertation will appreciate how good that suggestion was.

The research described in this dissertation was initiated at Cornell University, and then continued at Yale University for the past year and a half. At Yale, Professor Erin Lavik was unusually generous with not only her new research equipment, but also in sharing the battle to set up research labs in a fledgling department. The timing of her arrival on campus is proof that not all the things outside of my control at Yale were working against me.

In the lab, Hong Shen deserves special mention for the tough love required to teach a somewhat sloppy engineer how to do passable cell culture. I have also benefited greatly from having a number of talented students work with me on various aspects of this project. At Cornell, John Falcone was willing to take a chance with his senior thesis at a time when it wasn't yet certain that my ideas were at all workable. At Yale, Thomas Leung, Allison Carey, and Sara Royce all made significant

contributions to the work described here. The enthusiasm and energy these four students each brought to this project was always refreshing for me during the times when I was pessimistic.

Margaret Cartiera gets a paragraph of her own. I can't possibly begin to tell you how much I owe Margaret. I'm also not going to tell you how great she is – the more people who know, the more competition I will have when trying to recruit her to work with me again in the future. But she's pretty great. Trust me.

Away from the lab, I'd like to thank the classmates who kept me balanced with lunchtime card games, and also those who helped extend my amateur hockey career a few years longer than I thought it would last (and perhaps longer than it should have). Todd Schroeder, in addition to being part of both of the above groups, deserves special mention for his unfailing presence in Olin Hall on those Saturdays I found myself at the lab bench. His willingness to tolerate of my repeated cries of "Todd, it's Saturday. Go home!" over the years without once trying to kill me was but a small display of his remarkable ability to endure.

Finally, I thank Clara. A significant part of my love for Clara comes from recognition that she is many good things that I am not, including patient. She has listened to me say goodbye and then drive out of town before dawn far too many times over the past couple of years, and I am sorry. I'll be home soon, for good this time.

This work was supported by the National Institutes of Health and the Air Force Office of Scientific Research.

TABLE OF CONTENTS

Chapter 1: Introduction	1
1.1 Biodegradable microspheres for drug delivery	1
1.2 Targeting microspheres to specific cells	3
1.2.1 Targeting by nonspecific methods.....	4
1.2.2 Targeting by specific ligands on the microsphere surface	5
1.3 Increasing vaccine delivery to M cells – an application of microsphere targeting	6
1.4 Increasing capacity for surface ligands on PLGA microspheres.....	13
1.4.1 Microsphere production by the double-emulsion method.....	13
1.4.2 Current methods for affixing ligands to the surface of PLGA microspheres	16
1.4.3 A novel PLGA microsphere formulation with a high density of surface carboxylic acid groups	17
Chapter 2: PLGA microspheres with surface carboxylic acid groups	23
2.1 Production of PLGA microspheres	23
2.2 Comparison of morphology of different microsphere formulations	24
2.3 Conjugation of ligands to microspheres	28
2.4 Detection of conjugated ligands	29
2.5 Encapsulated FITC-BSA release assay	36
2.6 Conjugated ligand release assay	38
2.7 Summary of ligand conjugation to PLGA/PEMA microspheres	38

Chapter 3: <i>In vitro</i> evaluation of microsphere targeting by surface-conjugated ligands.....	41
3.1 Purpose	41
3.2 Retention of targeted microspheres on receptor-bearing agarose matrices	41
3.2.1 Conjugation of ligands to PLGA/PEMA microspheres with encapsulated FITC-BSA for targeting to agarose-bound receptors	42
3.2.2 Detection of conjugated ligands on microsphere surfaces	42
3.2.3 Conjugation of mouse IgG to agarose beads.....	44
3.2.4 Retention of Microspheres on Agarose Columns.....	44
3.3 Adhesion of targeted microspheres to Caco-2 cell monolayers	48
3.3.1 Preparation of PLGA/PEMA microspheres for monolayer targeting.....	48
3.3.2 Caco-2 cell culture.....	49
3.3.3 Microsphere Adhesion Study	51
3.3.4 Microsphere Counting.....	54
3.4 Conversion of Caco-2 monolayers to M cells	57
3.4.1 Coculture of Caco-2 monolayers with Raji B cells.....	58
3.4.2 Staining of monolayers.....	58
3.4.3 Microsphere uptake experiments with cocultured monolayers .	65
3.5 Summary of in vitro microsphere targeting experiments	68
Chapter 4: M cell targeted microspheres in mouse models	70
4.1 Purpose	70

4.2 Uptake of orally administered microspheres with encapsulated	
FITC-BSA in mouse tissues	71
4.2.1 Microsphere administration and tissue collection	71
4.2.2 Uptake study results	72
4.3 Targeting of antigen-carrying microspheres to mouse M cells	72
4.3.1 Production of PLGA/PEMA microspheres with	
encapsulated OVA for mouse M cell targeting	72
4.3.2 Determination of OVA loading level in microspheres	76
4.3.3 Immunization of mice with microspheres containing OVA	78
4.3.4 Collection and processing of samples for ELISA	79
4.3.5 Measurement of anti-OVA antibodies by ELISA	80
4.3.6 High-dose immunization of mice with OVA encapsulated	
in PLGA/PEMA microspheres	83
4.4 Conclusions for mouse M cell targeting studies	86
 Chapter 5: Conclusions and recommendations for future work	 88
5.1 Conclusions	88
5.2 Recommendations for future work	92
 References	 97

LIST OF FIGURES

Figure 1.1	Structure of PLGA.....	2
Figure 1.2	Obstacles to M cell targeting.....	10
Figure 1.3	Encapsulation of protein in PLGA microspheres by the double-emulsion method	14
Figure 1.4	Use of PVA in microsphere production	18
Figure 1.5	Replacing PVA with PEMA for microsphere production.....	20
Figure 1.6	Conjugation of ligands to PLGA/PEMA microspheres by carbodiimide chemistry	22
Figure 2.1	Scanning electron micrographs of microspheres.....	25
Figure 2.2	Microsphere size distributions.....	26
Figure 2.3	PLGA/PEMA microspheres with encapsulated FITC-BSA.....	27
Figure 2.4	Scanning electron micrograph of PLGA/PEMA microspheres after conjugation to 5-(aminoacetamido)fluorescein.....	30
Figure 2.5	Transmission and fluorescence micrographs of PLGA microspheres after conjugation to 5-(aminoacetamido)fluorescein.....	31
Figure 2.6	Dot plots of fluorescence intensity vs. forward scatter for PLGA/PEMA microspheres	32
Figure 2.7	Comparison of capacity for coupling of 5-(aminoacetamido)fluorescein to the surface of various microsphere formulations	33
Figure 2.8	Detection by immunocytometry of HSA and hIgA conjugated to PLGA/PEMA microspheres	35
Figure 2.9	Cumulative release of encapsulated FITC-BSA from PLGA microspheres during incubation in PBS at 37°C	37

Figure 2.10	Retention of conjugated 5-(aminoacetamido)fluorescein at the surface of PLGA/PEMA microspheres	39
Figure 3.1	Detection of goat-anti-mouse IgG, BSA, and streptavidin conjugated to the surface of PLGA/PEMA microspheres with encapsulated FITC-BSA.....	43
Figure 3.2	Retention of streptavidin-conjugated microspheres on agarose columns	46
Figure 3.3	Retention of goat-anti-mouse IgG-conjugated microspheres on agarose columns	47
Figure 3.4	Detection of UEA 1 conjugated to the surface of PLGA/PEMA microspheres with encapsulated FITC-BSA	50
Figure 3.5	Caco-2 cell culture on Transwell™ filters	52
Figure 3.6	Transepithelial electrical resistance measurements for Caco-2 cells grown on Transwell™ filters	53
Figure 3.7	PLGA/PEMA microspheres adhering to the apical surface of Caco-2 cell monolayers.....	55
Figure 3.8	Effect of surface ligands on microsphere adhesion to Caco-2 monolayers.....	56
Figure 3.9	hIgA receptor expression on Caco-2 monolayers 21 days after culture	60
Figure 3.10	Sialyl Lewis A antigen expression on Caco-2 monolayers 21 days after culture	61
Figure 3.11	Selective expression of sialyl Lewis A antigen on Caco-2 cell apical surfaces.....	62
Figure 3.12	hIgA receptor expression on Caco-2 monolayers after coculture with 2×10^5 Raji B cells	63

Figure 3.13	Sialyl Lewis A antigen expression on Caco-2 monolayers after coculture with 2×10^5 Raji B cells	64
Figure 3.14	Degradation of Caco-2 monolayers after coculture with 2×10^6 Raji B cells	66
Figure 3.15	Effect of Raji B cells on electrical resistance of Caco-2 monolayers ...	67
Figure 3.16	Transcytosis of PLGA/PEMA microspheres across Caco-2 monolayers cocultured with 2×10^6 Raji B cells	69
Figure 4.1	Fluorescence intensity of PLGA/PEMA microspheres with encapsulated FITC-BSA.....	73
Figure 4.2	Mouse tissues analyzed for microsphere uptake by flow cytometry.....	74
Figure 4.3	PLGA/PEMA microspheres with encapsulated OVA.....	75
Figure 4.4	Detection of UEA 1 and BSA conjugated to the surface of PLGA/PEMA microspheres with encapsulated OVA.....	77
Figure 4.5	ELISA results for mouse samples collected 7 weeks after immunization.....	82
Figure 4.6	ELISA results for mouse samples collected 12 weeks after immunization.....	84
Figure 4.7	Anti-OVA response to high doses of OVA encapsulated in PLGA/PEMA microspheres	85

LIST OF TABLES

Table 1.1.	Mucosal and Systemic Immune Responses to Orally Delivered Microsphere Vaccines	7
Table 4.1	Summary of Immunizations with Targeted PLGA/PEMA Microspheres with Encapsulated OVA	79

LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
EDC	1-Ethyl-3-(3-dimethylaminopropyl)- carbodiimide
FBS	Fetal Bovine Serum
FITC-BSA	Fluorescein isothiocyanate-conjugated BSA
HBSS	Hank's Balanced Salt Solution
hIgA	Human IgA
HSA	Human Serum Albumin
MLN	Mesenteric Lymph Node
OVA	Ovalbumin
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PEG	Poly(ethylene glycol)
PEMA	Poly(ethylene-co-maleic acid)
PLA	Poly(lactic acid)
PLGA	Poly(lactic-co-glycolic acid)
PLGA-COOH	PLGA with carboxylic acid end groups
PVA	Poly(vinyl alcohol)
SLAA	Sialyl Lewis A Antigen
UEA 1	<i>Ulex europaeus</i> agglutinin 1

CHAPTER 1 INTRODUCTION*

1.1 Biodegradable microspheres for drug delivery

Over the past 25 years, biodegradable polymer microspheres have been investigated for a wide variety of drug delivery applications, including but not limited to vaccines [1], tumor treatment [2], drug delivery to the retina [3], and control of inflammation [4]. Entrapment of drugs within microspheres provides many advantages compared to more traditional drug formulations. The microspheres release encapsulated molecules over extended time intervals, from days to several months. Release rates can be modified by adjustment of microsphere physical properties such as polymer molecular weight, monomer composition, and microsphere size. Microspheres provide a dry, stable environment for encapsulated molecules, extending the shelf life of the drug and making refrigerated storage less critical. The microspheres continue to protect the encapsulated agent after administration, permitting delivery by routes that might otherwise not be feasible. For example, microspheres can be used to orally administer agents that might be degraded by exposure to acid or digestive enzymes in the upper gastrointestinal tract.

The most commonly used polymer in biodegradable microsphere formulations is PLGA. The lactic and glycolic acid monomer units of PLGA are linked by ester bonds (Figure 1.1). Degradation of PLGA occurs by hydrolysis of these ester bonds, leading to a decrease in molecular weight of the polymer. This degradation occurs throughout the volume of the microspheres, and is called bulk erosion. The microspheres maintain their structure during degradation, until PLGA oligomers

* Sections of this chapter originally appeared in Keegan, M. E.; Whittum-Hudson, J. A.; Saltzman, W. M. *Biomaterials* **2003**, *24*, 4435-4443.

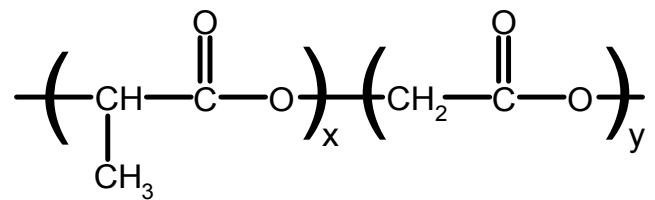


Figure 1.1. Structure of PLGA. Lactic acid (x) and glycolic acid (y) groups are linked in random order by ester bonds. Relative monomer composition (ratio of x to y) can be adjusted as desired during polymerization.

become short enough that they become soluble in the surrounding aqueous environment. At this point, the microspheres begin to physically break apart. Degradation of the PLGA oligomers continues until these oligomers are completely hydrolyzed to lactic and glycolic acid monomer units. These nontoxic monomers are found naturally in cells as part of normal cell metabolism, eventually being metabolized to carbon dioxide and water [5], which is a significant advantage to using PLGA for biomedical applications such as drug delivery in which biocompatibility of the material is crucial.

1.2 Targeting microspheres to specific cells

Biodegradable microspheres are of interest in many applications in part because they allow for localized delivery of the encapsulated agent directly to the desired site. Delivering therapeutic agents directly to the site of interest instead of systemically can result in lower required doses to achieve the desired effect, which has advantages in terms of both cost savings and reduction of potential unwanted side effects. Often this site specificity comes from direct physical administration of the microspheres at the site of interest (such as injection into the center or periphery of tumors) [6]. Oral or intravenous administration of the microspheres is simpler, but with the trade off of loss of site specificity. Efforts to solve the problem of maintaining site specificity for microspheres delivered by mucosal or intravenous routes involve modifying the surface properties of the microspheres to increase their bioadhesiveness to desired tissues [7], through changes to microsphere properties and addition of surface ligands which bind to receptors on the surface of targeted cells.

1.2.1 Targeting by nonspecific methods

Physicochemical properties of microspheres influence their interactions with tissues. For example, negatively charged microspheres have reduced uptake in the intestine compared to non-ionic microspheres [8]. This result is not surprising considering that the intestinal epithelial lining is also negatively charged, due at least in part to terminal sialic acid groups on side chains of secreted mucins and membrane-bound glycoproteins [9]. The permeability of microspheres through (negatively charged) gastric mucus decreases as the ζ -potential of the microspheres becomes increasingly negative [10].

In addition to the effect of surface charge, the hydrophobicity of microspheres also affects their interactions with tissues. Intestinal uptake studies with PLGA and polystyrene microspheres show that the more hydrophobic polystyrene microspheres have a much higher level of uptake [11,12]. The correlation of particle uptake with hydrophobicity was verified with a panel of microspheres made of various polymers with significantly different hydrophobicities [11]. For nasal administration, however, microspheres made of a PLA-PEG diblock copolymer demonstrated an approximately 10-fold increase in uptake into the circulation and 4-fold increased uptake into lymph nodes in rats compared to microspheres of PLA [13]. Incorporation of PEG into the formulation makes the microspheres more hydrophilic, so this result runs counter to the findings for uptake in the intestine and suggests that microsphere physicochemical properties need to be optimized for the particular delivery route being considered.

For microspheres delivered intravenously, incorporating PEG into the formulation increases the circulation time of the microspheres [14,15]. Clearance of micro- and nanospheres from the systemic circulation by the liver and spleen can interfere with effective use of these vehicles for sustained-release drug delivery in the bloodstream. Contrary to the efforts to target microspheres to specific cells or tissues,

using PEG to reduce the clearance rate is an “anti-targeting” application, in which microsphere interactions with tissues are undesirable and microsphere properties are altered to minimize those interactions. The effects of changes to microsphere physicochemical properties on their interactions with cells and tissues are limited in specificity. Efforts to optimize these properties for a given application are best suited to situations in which the goal is a general increase (or decrease) in microsphere adherence or uptake without regard for any specific cell types.

1.2.2 Targeting by specific ligands on the microsphere surface

Beyond the general tissue targeting effects that can be achieved by altering microsphere physicochemical properties, site specific targeting can be achieved through ligand-receptor interactions. By labeling the surface of microspheres with appropriate ligands, surface receptors on selected cell types can be targeted. When the microspheres encounter cells expressing the correct surface receptors, binding of the receptors to ligands on the microsphere surface will cause retention of the microsphere at the cell surface. This strategy is being investigated for a number of different applications, including tumor targeting [16] and increasing uptake of drugs into the circulation by adhesion of microspheres to enterocytes of the intestinal epithelium [17,18]. A more sophisticated application of ligand-receptor targeting of microspheres involves mimicry of the rolling and firm adhesion behavior demonstrated by leukocytes when they encounter sites of inflammation in the systemic circulation [19]. Microspheres with surface-bound sialyl Lewis X antigen were evaluated in parallel-plate flow chambers coated with P- and E-selectin, which are expressed by vascular endothelial cells as part of the inflammatory response. Rolling velocity of the microspheres in the flow chamber decreased with increasing density of conjugated sialyl Lewis X antigen [20].

The concept of using specific ligand-receptor binding interactions to target microspheres to specific cell types draws support from observations of pathogen interactions with cells of the intestinal epithelium. Sampling of intestinal contents for presentation to the immune system is performed by M cells, which are specialized epithelial cells capable of taking up particles. M cells are present in the epithelium overlying lymphoid follicles, and transcytose particles rapidly from the intestinal lumen to the underlying lymphoid tissue [21]. A variety of different pathogens, both viral and bacterial, have the ability to target and selectively bind to M cells in the gut [22]. This targeting is accomplished by interactions of ligands on the pathogen surfaces with receptors on the apical surface of the M cells. The pathogens take advantage of the particle uptake behavior of the M cells to penetrate the epithelial lining of the intestine and initiate infection. It is important to note that the short list of licensed oral vaccines includes the Sabin attenuated oral polio vaccine as well as an attenuated *Salmonella typhi* vaccine. These pathogens are among those with demonstrated ability to target M cells [23,24]. This observation suggests that the addition of appropriate ligands to the surface of microspheres would significantly increase the effectiveness of orally administered vaccines encapsulated in microspheres.

1.3 Increasing vaccine delivery to M cells – an application of microsphere targeting

A number of studies in animal models have demonstrated that oral delivery of antigen-containing microspheres leads to both systemic and mucosal immune responses (Table 1.1). These immune responses, however, are typically much lower in strength than those obtained by injection of microspheres, and require much larger doses of antigen. The difference in dose and effectiveness is vast: one investigator has summarized the difference by remarking that a 100-fold increase in the oral dose will

Table 1.1. Mucosal and Systemic Immune Responses to Orally Delivered Microsphere Vaccines

Antigen(s)	Antigen dose and schedule	Observed immune response	Ref.	Notes
Ovalbumin	Six 100 µg doses over 10 days	Systemic CTL Intestinal IgA	[25]	
	250 µg/day for 3 days; single 250 µg booster at 4 weeks	Intestinal IgA Serum IgG	[26]	
	1 mg/day for 3 days; repeated 4 weeks later	Salivary IgA Vaginal IgA Intestinal IgA Serum IgG, IgA	[27]	Expands on similar findings in [28]
Influenza vaccine	Single 70 µg dose	Salivary IgA Intestinal IgA	[29]	
Bordetella pertussis	Single 10 µg dose of fimbriae	Serum IgM, IgG, IgA Salivary IgA, IgG Fecal IgA, IgG Vaginal IgG	[30]	>95% reduction in viable bacteria after intranasal challenge compared to unimmunized controls
	Three 100 µg doses of pertussis toxoid and filamentous haemagglutinin over 8 weeks	Serum IgG, IgA	[31]	Complete clearance of bacteria 14 days after intranasal challenge
Yersinia pestis V and F1 antigens	3.0 µg V antigen 0.47 µg F1 antigen in single dose	Serum IgG Intestinal IgA Bronchial IgG	[32]	Intranasal booster significantly enhanced anti-V antigen response
Anti-idiotypic to chlamydial exoglycolipid antigen	Two 4 µg doses over 2-3 weeks	Serum neutralizing antibody	[33]	Up to 90% reduction in infectious yield after challenge compared to controls

produce a 100-fold smaller immune response in comparison to an injected dose of the same microspheres [34].

So while vaccines made with biodegradable microspheres have been shown to be capable of generating immune responses, the low efficiency of these particles when delivered orally remains a challenge. This is underscored by the results of a human trial in which antigens of enterotoxigenic *Escherichia coli* were encapsulated in PLGA and then administered by intestinal tube. Despite receiving 4 large (1 mg) doses of the encapsulated antigen over a 4-week period, only 30% of the volunteers were protected from a subsequent *E. coli* challenge [35]. A principal cause of the low efficiency of orally administered microsphere vaccine formulations is thought to be poor uptake of the particles by the relevant tissues of the intestine. If this is the case, mimicking the behavior of pathogens with M cell targeting ligands could increase uptake efficiency and lead to improved immune responses to vaccines encapsulated in microspheres.

The first step in targeting microspheres to M cells (or any other cell type) is identification of ligands that bind specifically to the selected cells. The lectin UEA 1 binds to mouse M cells, but not enterocytes or mucus-secreting goblet cells [36]. To test the effect of lectin-mediated targeting, UEA 1 has been conjugated to liposomes which were then delivered orally [37] or administered directly to the small intestine [38] of mice. Addition of the lectin increased the uptake of these liposomes by approximately three-fold compared to untargeted liposomes. UEA 1 conjugated to the surface of carboxylated polystyrene microspheres increased microsphere uptake after oral delivery to mice by nearly 100-fold [39]. Most ligand-M cell specific binding interactions appear to be species-specific, however, and no lectin has been found to date that binds specifically to human M cells but not to regular enterocytes. Screening of various ligands has indicated, however, that human M cells preferentially express

the sialyl Lewis A antigen, as determined by binding of a monoclonal antibody specific for this antigen to explants of human intestinal tissue [40].

Microspheres coated with secretory IgA have roughly 20-fold increased uptake in mice relative to microspheres coated with BSA [41]. M cells have been shown to selectively bind IgG and IgA, irrespective of the antigen specificity of the immunoglobulin molecule [42]. Unlike lectin-M cell binding, this antibody selectivity is not species-specific, as human secretory IgA was shown to increase particle uptake in mice [41] and a mouse monoclonal IgA demonstrated binding selectivity for M cells in the intestinal epithelia of not only mice but also rats and rabbits [42].

Even if appropriate ligands can be identified and conjugated to the surface of microspheres, potential obstacles to effective targeting need to be overcome. Although many of these obstacles apply to just about any *in vivo* targeting scenario, the particular application of targeting vaccines to M cells provides an opportunity to illustrate a number of them. A variety of events can occur either during preparation or after administration of targeted oral microsphere vaccines that could interfere with targeting of the microspheres to M cells (Figure 1.2). First, the linkages between targeting molecules and the microspheres must remain intact until the microspheres reach their target (Figure 1.2B). The acidic environment of the stomach is of particular concern in this regard for M cell targeting, as the degradation of PLGA is acid-catalyzed. The premature release of targeting molecules is significant not only because the released molecules can no longer bind the microspheres to their target, but also because they will serve as targeting inhibitors, by competing for binding sites with targeting molecules still bound to microspheres. In one investigation of the release of surface bound lectin from microspheres into HEPES buffer at 4°C, after 21 days less than 10% of the lectin had been released from the microspheres [17]. The effect of exposure to acid on release of surface-conjugated molecules, however,

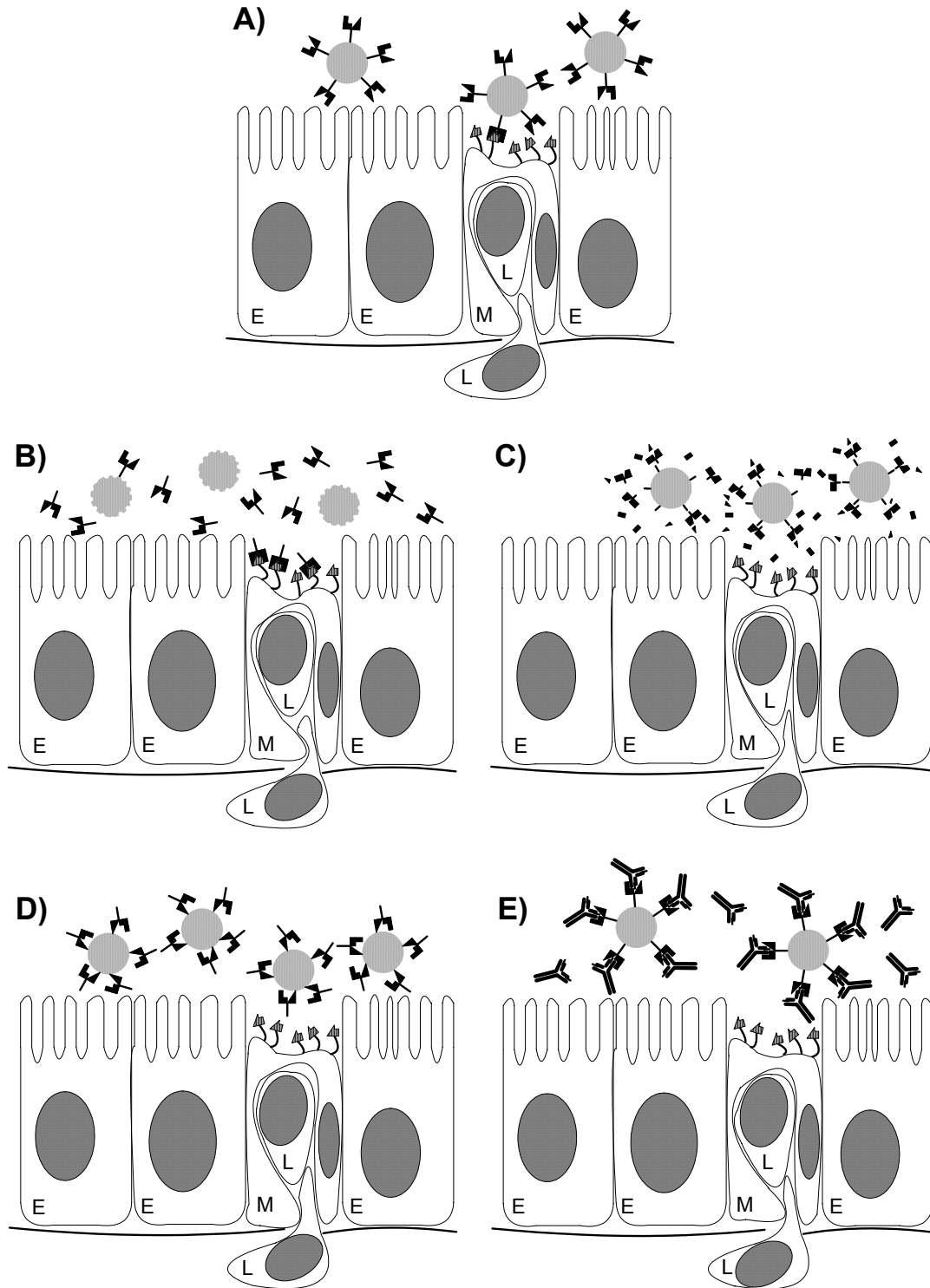


Figure 1.2. Obstacles to M cell targeting. A: Successful targeting of M cell receptors. B: Premature release of targeting ligand. C: Enzymatic degradation of targeting ligand. D: Microsphere-ligand coupling near ligand binding site. E: Antibody response to targeting ligand. E = Enterocyte; L = Lymphocyte; M = M cell.

remains to be determined. Should microsphere degradation due to stomach acidity prove to be a problem, the solution might be as simple as co-administration of an appropriate buffer with the microspheres.

Not only does the linkage between targeting molecule and microsphere need to remain stable, but the selected targeting molecules must also be resistant to degradation in their *in vivo* environment (Figure 1.2C). The need to protect proteins from rapid degradation in the digestive tract, for example, is a primary reason why encapsulating vaccines within microspheres is desirable; surface-conjugated proteins however, will not enjoy this same protection. Lectins display good resistance to degradation [43], but other potential targeting molecules (such as the monoclonal specific to sialyl Lewis A antigen) remain unproven in this regard. Secretory IgA may prove effective as a stable M cell targeting molecule; dimeric IgA coupled to secretory component demonstrates good resistance to degradation in the intestinal lumen [44]. Other molecules that should demonstrate suitable resistance to degradation are the targeting ligands used by pathogens to selectively bind to M cells. A subunit of invasins, an outer membrane protein of *Yersinia pseudotuberculosis*, was produced in *E. coli* culture, purified, and coupled to functionalized polystyrene microspheres. These targeted microspheres showed a six-fold increase in uptake into the systemic circulation compared to controls after oral administration to rats [45]. Future work to identify, isolate, and produce in culture the M cell targeting ligands of other pathogens could provide a library of gastrointestinal tract-stable molecules that could be used to direct vaccine-carrying microspheres to M cells.

Depending on the functional groups present on the surface of the microspheres, a number of different conjugation schemes can be employed for linking ligands to microspheres. These different schemes use different functional groups on the ligand molecule. Different conjugation schemes may prove optimal for different targeting

molecules, depending on what functional groups are present in or around the receptor binding region of the targeting ligand. The method by which targeting ligands are fixed to the microspheres must not disrupt the ability of the ligand to bind to its target receptor. If the targeting molecule is conjugated to the microsphere at a site close to the receptor binding region, steric hindrance could prevent interaction between the targeting molecule and cells being targeted (Figure 1.2D).

Beyond factors related to the means by which targeting molecules are fixed to the microspheres, another issue that will require investigation is the potential for immune responses to be generated against the targeting molecules themselves (Figure 1.2E). For M cell targeting, the presence in the gut of antibodies specific for targeting ligands could neutralize the targeting ability of the microspheres. Intestinal antibody interference with vaccine immunogenicity has been observed clinically with the *S. typhi* strain Ty21a oral vaccine [46]. The potential for a similar anti-targeting ligand response would be of particular concern for any oral vaccine regimen requiring multiple administrations of microspheres using the same targeting ligand. Mice generate a mild anti-UEA 1 response when the lectin is orally delivered, for example [47], although at this point it remains unclear just how strong a mucosal anti-M cell ligand response would be required to interfere with targeting of subsequent doses of microspheres using that same ligand. Different targeting ligands would be likely to provoke immune responses of varying intensity. Of the current targeting ligands being investigated, human secretory IgA should be the most suitable for use in repeated administrations, as it is normally present in the intestinal lumen and should therefore be well-tolerated by the immune system as a native protein.

1.4 Increasing capacity for surface ligands on PLGA microspheres

Understanding the process by which PLGA microspheres are produced is critical to efforts to increase the microsphere surface capacity for conjugated ligands. Microsphere properties depend on a variety of processing variables, providing a number of opportunities to alter production to create microspheres with the desired characteristics.

1.4.1 Microsphere production by the double-emulsion method

PLGA microspheres are commonly fabricated by an emulsion method. This technique is straightforward and adaptable for encapsulating molecules with a range of physical properties. For encapsulating hydrophilic molecules such as proteins, a double emulsion technique is used, as summarized in Figure 1.3. First, PLGA is dissolved in an appropriate organic solvent, such as dichloromethane. Added to this polymer solution is the molecule to be encapsulated, in aqueous solution. The volume of the aqueous phase should be as small as possible, ideally no more than 10% of the volume of the PLGA solution. The two phases are then emulsified, by either high shear mixing or sonication. The resulting primary emulsion contains small droplets of the aqueous drug solution within the organic PLGA solution. Next, an aqueous surfactant solution (most commonly PVA) is added to the primary emulsion. The volume of the surfactant solution should be at least twice as great as the volume of the organic phase, but small enough that the total volume can be rapidly processed by the equipment used for emulsification. The mixture is then emulsified again, to create the secondary emulsion. This emulsion contains two dispersed phases, with the inner dispersed phase consisting of droplets of the molecule to be encapsulated. The inner dispersed phase is contained within the outer dispersed phase, which is the organic PLGA solution. The outer dispersed phase exists as droplets in the continuous phase

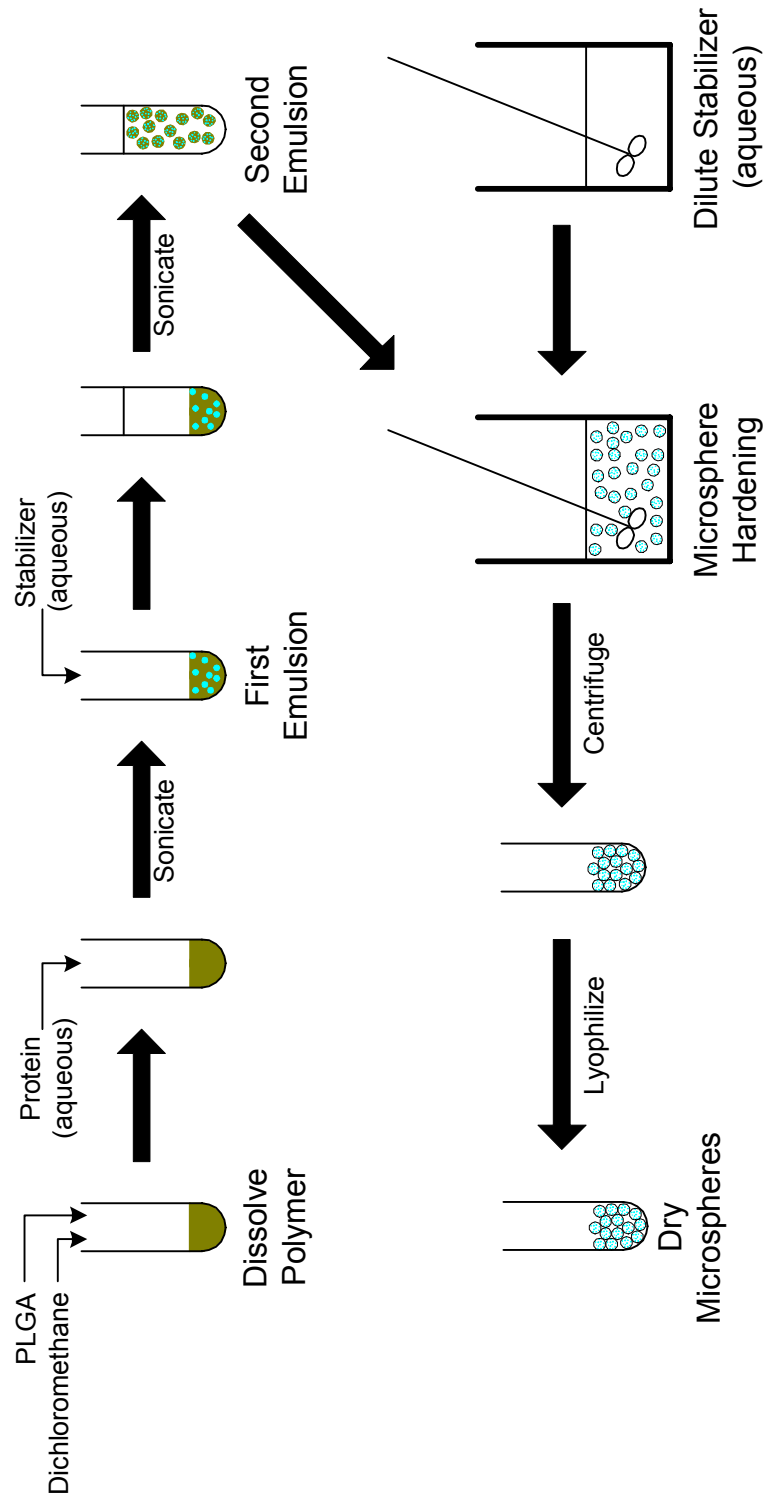


Figure 1.3. Encapsulation of protein in PLGA microspheres by the double-emulsion method.

of the aqueous surfactant solution. Surfactant molecules partition at the interface of the continuous phase and outer dispersed phase, with hydrophilic regions in the continuous phase and hydrophobic regions in the dispersed phase. The surfactant molecules prevent coalescence of the emulsified droplets of the organic phase.

The emulsion is quickly transferred to a large volume of aqueous surfactant solution, which is rapidly stirred to disperse the droplets of the organic phase. The volume of surfactant solution used is enough to fully dissolve the organic solvent, and therefore depends on the solubility of the organic solvent in water. For dichloromethane, which is soluble in water up to approximately 2% [48], 100 ml of stirring surfactant solution is used for every 2 ml dichloromethane. When the emulsion is transferred to the stirring surfactant solution, the organic solvent droplets rapidly dissolve into the solution. The water-insoluble PLGA precipitates out of solution, forming microspheres in the place of dissolving organic phase droplets and entrapping the molecules of the inner dispersed phase. The newly formed microspheres are stirred for several hours to evaporate away the organic solvent, are washed several times with water, and then lyophilized to form a free-flowing dry powder.

To encapsulate hydrophobic compounds into PLGA microspheres, the compound of interest is simply dissolved directly into the initial organic PLGA solution. Only one emulsification is performed, creating a dispersed organic phase containing PLGA and the molecule to be encapsulated within the continuous aqueous phase of the surfactant solution. After this emulsification, processing continues identically to the double-emulsion method.

1.4.2 Current methods for affixing ligands to the surface of PLGA microspheres

Most studies of particle targeting to cells by specific ligands have avoided the use of biodegradable polymer microspheres. Functionalized polystyrene microspheres are commercially available, with well-established protocols for coupling molecules to their surfaces. Similarly, liposomes can be formulated from molecules with a variety of different reactive groups suitable for coupling to targeting ligands. Reports of chemical coupling to PLA or PLGA microspheres, however, are much less prevalent. The commonly used forms of these polymers are chemically (and biologically) inert, with the exception of the degradation reaction. This inertness is a desirable property from a biocompatibility standpoint, but complicates attempts to chemically couple molecules to PLGA microspheres. Furthermore, coupling reactions must be performed under conditions designed to minimize premature degradation of the microspheres and their contents.

Despite the difficulties associated with conjugating molecules to the surface of biodegradable microspheres, recent reports indicate that there are a number of ways by which it can be accomplished. For example, spray dried microspheres made of PLGA-COOH [49] have had lectins conjugated to their surface by carbodiimide conjugation [17]. This conjugation was achieved by amide bond formation between PLGA carboxylic acid groups and primary amines present on the lectins.

Direct coupling to PLGA carboxylic acid end groups may not be possible, however, for microspheres made by emulsion methods. Microspheres produced by emulsion methods have surface layers of surfactant molecules. During microsphere formation, as organic solvent dissolves out of droplets into the continuous phase, PLGA precipitating out of solution entraps surfactant molecules present at the interface between the continuous and organic dispersed phases. The hydrophilic portion of the surfactant does not partition into the organic phase, and is therefore left

exposed at the microsphere surface. When PVA is used as the surfactant, the result is microspheres with surface hydroxyl groups, which are present as side groups on PVA chains (Figure 1.4). This surfactant layer remains at the microsphere surface even after repeated water washing of the microspheres [50], and provides a steric barrier which may interfere with successful conjugation of ligands to PLGA-COOH in the microsphere core. In this situation, glutaraldehyde can be used to conjugate proteins to PVA hydroxyl groups present on the surface of microspheres [51].

Another method for coupling ligands to PLGA takes advantage of biotin-avidin interactions. Copolymers of PLA-PEG were produced which have biotin molecules conjugated to the ends of the polymer chains. This copolymer was used to produce nanospheres with biotin groups at the surface [52]. The nanospheres were then incubated with avidin, which exhibits strong, multivalent binding with biotin. Nanospheres were then incubated with biotinylated lectins, which was bound to the avidin present at the microsphere surface. In a similar method, PLGA microspheres can be incubated with copolymers of poly(L-lysine)-PEG-biotin [53]. The positive charge of the copolymer causes adsorption to the microsphere surface, which can be followed by incubation with avidin and biotinylated ligands as described above.

1.4.3 A novel PLGA microsphere formulation with a high density of surface carboxylic acid groups

The majority of studies performed to date using surface-conjugated ligands to target microspheres to tissues used carboxylated polystyrene microspheres [18,39,54-56]. These microspheres are ideal model particles because they have a high density of surface carboxylic acid groups at the microsphere surface, enabling a high level of surface ligand conjugation. Carboxylic acid groups can be linked to primary amines by use of carbodiimide cross-linkers by well-established protocols [57]. Carbodiimide

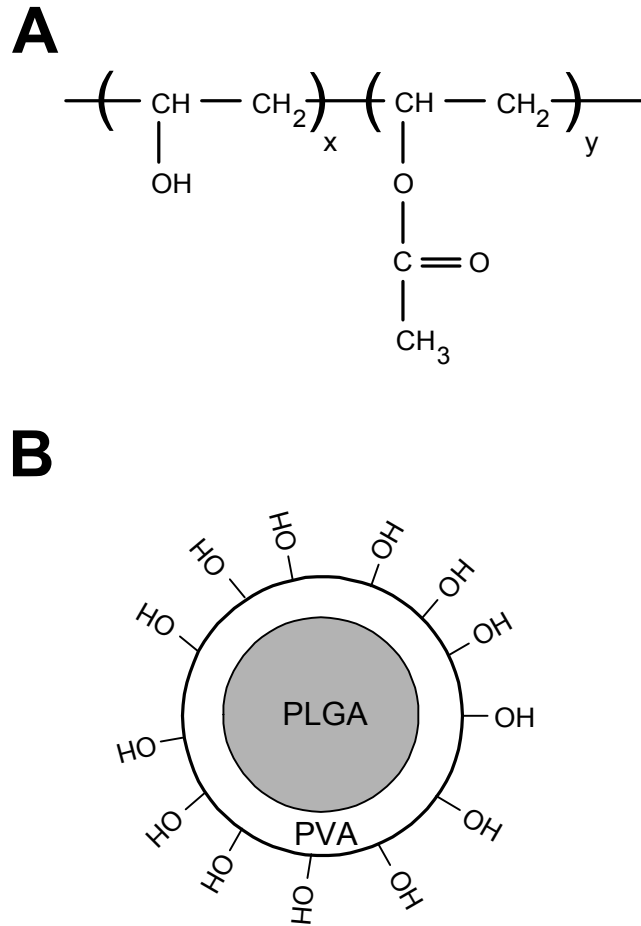


Figure 1.4. Use of PVA in microsphere production. A: Structure of PVA molecule. Groups with hydroxyl side chains (x) typically comprise from 88 to 100% of the monomer units. B: Stabilizing the microsphere emulsion with PVA results in microspheres with surface hydroxyl groups

conjugation is a two-step process, in which the carboxylic acid groups are first activated for cross-linking, followed by exposure to the primary amine-bearing compound and subsequent amide bond formation. The stepwise nature of this protocol and the chemistry of carbodiimides limits reactions to the desired carboxylic and amine functional groups. This is a substantial advantage of this cross-linking scheme compared to other cross-linking agents such as glutaraldehyde, which reacts with a variety of different chemical groups and therefore increases the chances of altering the structure of conjugated ligands at locations critical to their binding activity. Carbodiimide chemistry is also popular for biomolecule conjugation because all the processing steps take place in aqueous buffers, unreacted cross-linker degrades in water in a period of hours, and the amide linkages that are formed have good long-term stability in water. But while carboxylated polystyrene microspheres are ideal for ligand conjugation, these nondegradable particles are unsuitable for drug delivery.

PLGA microspheres with carboxylic acid groups at their surfaces would combine the ability to conjugate ligands to the microspheres by carbodiimide chemistry with a controlled release delivery vehicle of clinical relevance. In selecting a method for introducing carboxylic acid groups at the surface of PLGA microspheres consideration must be given to minimizing changes to other microsphere properties. Replacing the stabilizer PVA with a surfactant that has carboxylic acid side chains results in PLGA microspheres with surface carboxylic acid groups. PEMA is such a surfactant, which is structurally similar to PVA (Figure 1.5A) with the exception that PEMA has carboxylic acid side groups instead of hydroxyl side groups. Both surfactants are water-soluble polymers with backbones of carbon-carbon bonds and side chains of hydrophilic groups. PLGA microspheres made with PEMA as the stabilizer (PLGA/PEMA microspheres) (Figure 1.5B) achieve the goal of creating a biodegradable microsphere formulation with surface carboxylic acid groups while

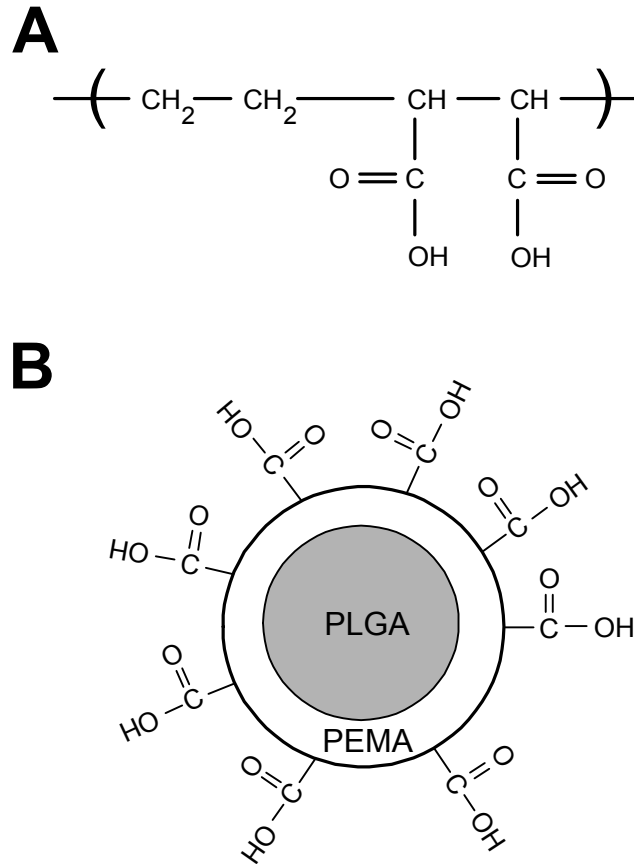


Figure 1.5. Replacing PVA with PEMA for microsphere production. A: Structure of PEMA molecule. B: Using PEMA as the stabilizer produces microspheres with surface carboxylic acid groups.

making no other significant changes to the microsphere fabrication method. This dissertation describes studies comparing PLGA/PEMA microspheres to other PLGA microsphere formulations, including the relative capacity of these microspheres for ligands conjugated to the surface by carbodiimide chemistry (Figure 1.6). The effect of surface-bound ligands on microsphere behavior in both *in vitro* and *in vivo* model systems is also evaluated.

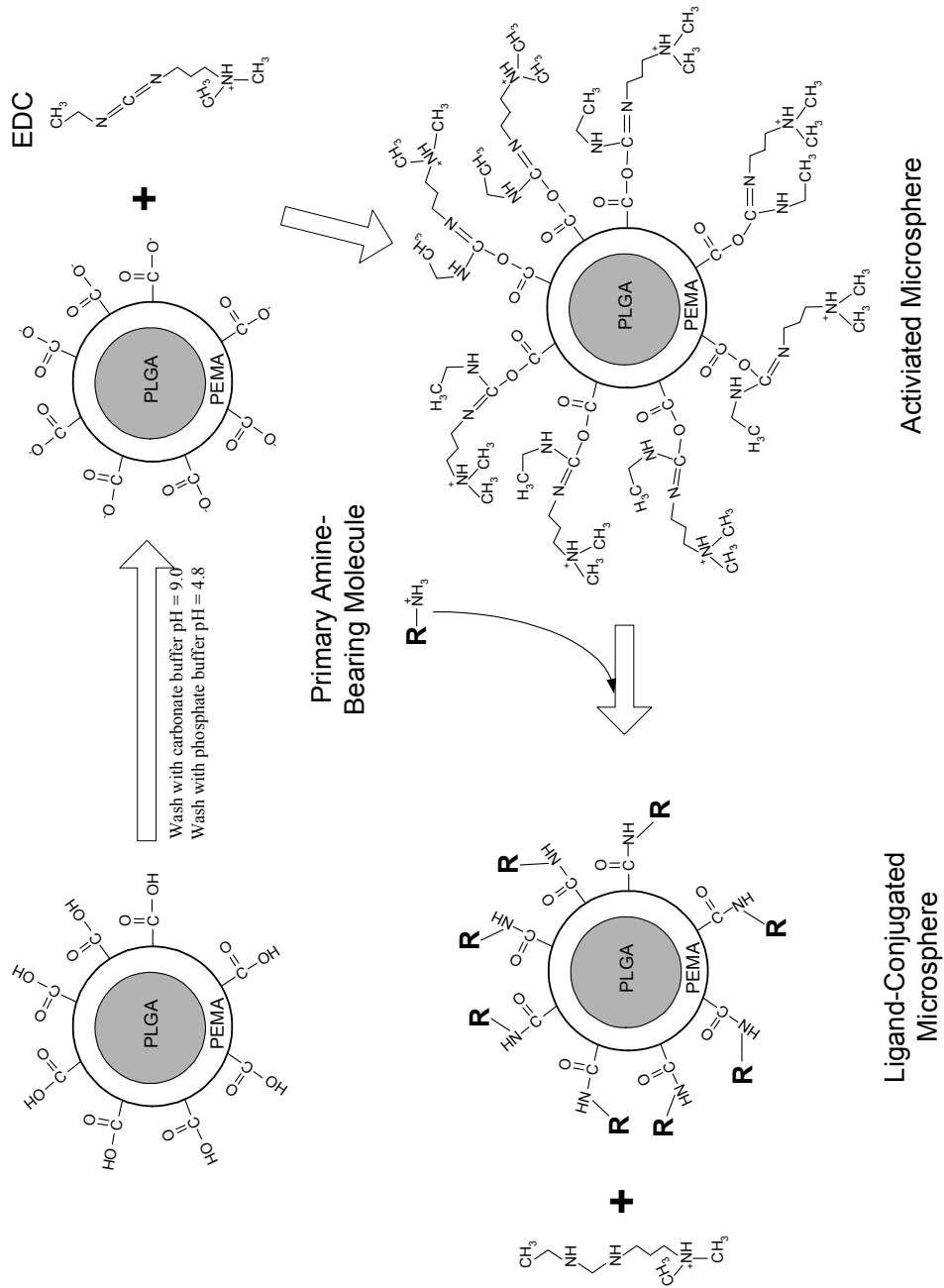


Figure 1.6. Conjugation of ligands to PLGA/PEMA microspheres by carbodiimide chemistry.

CHAPTER 2

PLGA MICROSPHERES WITH SURFACE CARBOXYLIC ACID GROUPS

2.1 Production of PLGA microspheres

Microspheres were prepared using the double-emulsion technique described in section 1.4.1, using PLGA with a 50:50 lactide:glycolide ratio and an inherent viscosity of 0.59 dL/g in hexafluoroisopropanol, and PLGA-COOH with a 50:50 lactide:glycolide ratio and an inherent viscosity of 0.32 dL/g in chloroform (Birmingham Polymers, Birmingham, AL). Four hundred milligrams of PLGA or PLGA-COOH was dissolved in 2 ml dichloromethane in a glass tube. One hundred microliters of a 75 mg/ml solution of FITC-BSA (Sigma, St. Louis, MO) in water was added to the polymer solution while gently vortexing the tube. For microspheres with no encapsulated protein, 100 μ l of Milli-Q water instead of FITC-BSA solution was added to the polymer solution. The polymer solution was then sonicated for 15 s at 40% amplitude with a TMX 400 sonic disruptor (Tekmar, Cincinnati, OH), to create the primary emulsion. Four milliliters of an aqueous 1% w/v solution of either PVA (Sigma) or PEMA (Polysciences, Warrington, PA) was added to the tube and the sonication step was repeated. Immediately after the second sonication, the emulsion was poured into 100 ml of 0.3% w/v aqueous solution of the same stabilizer used for the second emulsion, under rapid stirring with a magnetic stirrer. The resulting microspheres were stirred in this solution for 3 hours in order to evaporate away the dichloromethane. The microspheres were then washed 3 times, by centrifuging at 10,000x g for 10 minutes at 4°C and replacement of the supernatant with fresh Milli-Q water. Following the washes, microspheres were resuspended in 4 ml Milli-Q water and lyophilized to dryness.

2.2 Comparison of morphology of different microsphere formulations

Microspheres were fixed to aluminum sample stubs with double-sided carbon tape, and sputter coated with gold for viewing by scanning electron microscopy. Micrographs were analyzed with Scion Image software (Scion Corporation, Frederick, MD) to determine size distributions of different microsphere samples.

Switching from PVA to PEMA as the stabilizer for PLGA microsphere production did not noticeably change microsphere morphology. Both PLGA/PVA and PLGA/PEMA microsphere types exhibited smooth, unbroken surfaces (Figure 2.1), and nearly identical size distributions (Figure 2.2), with a mean diameter of 686 nm for PLGA/PVA, and 682 nm for PLGA/PEMA. The PLGA-COOH/PVA microspheres also had smooth, unbroken surfaces, but were noticeably smaller, with a mean diameter of only 361 nm. During PLGA-COOH/PVA microsphere production, undissolved polymer was found in the tube after the sonication steps. Microsphere yield calculations indicated that the PLGA-COOH had only dissolved to a concentration of approximately 150 mg/ml dichloromethane, compared to the 200 mg/ml used for making both PLGA/PVA and PLGA/PEMA microspheres. With this reduced polymer concentration comes a reduction in solution viscosity, resulting in more effective droplet dispersion during the sonication steps, and therefore smaller microspheres [58].

The presence of encapsulated protein did not significantly effect the size of PLGA/PEMA microspheres. Microspheres with encapsulated FITC-BSA (Figure 2.3A) have the same smooth, unbroken surfaces as microspheres with no encapsulated protein (Figure 2.1B). The size distributions are also very similar (Figure 2.3B), with the presence of FITC-BSA slightly increasing the mean microsphere diameter, from 682 nm to 813 nm.

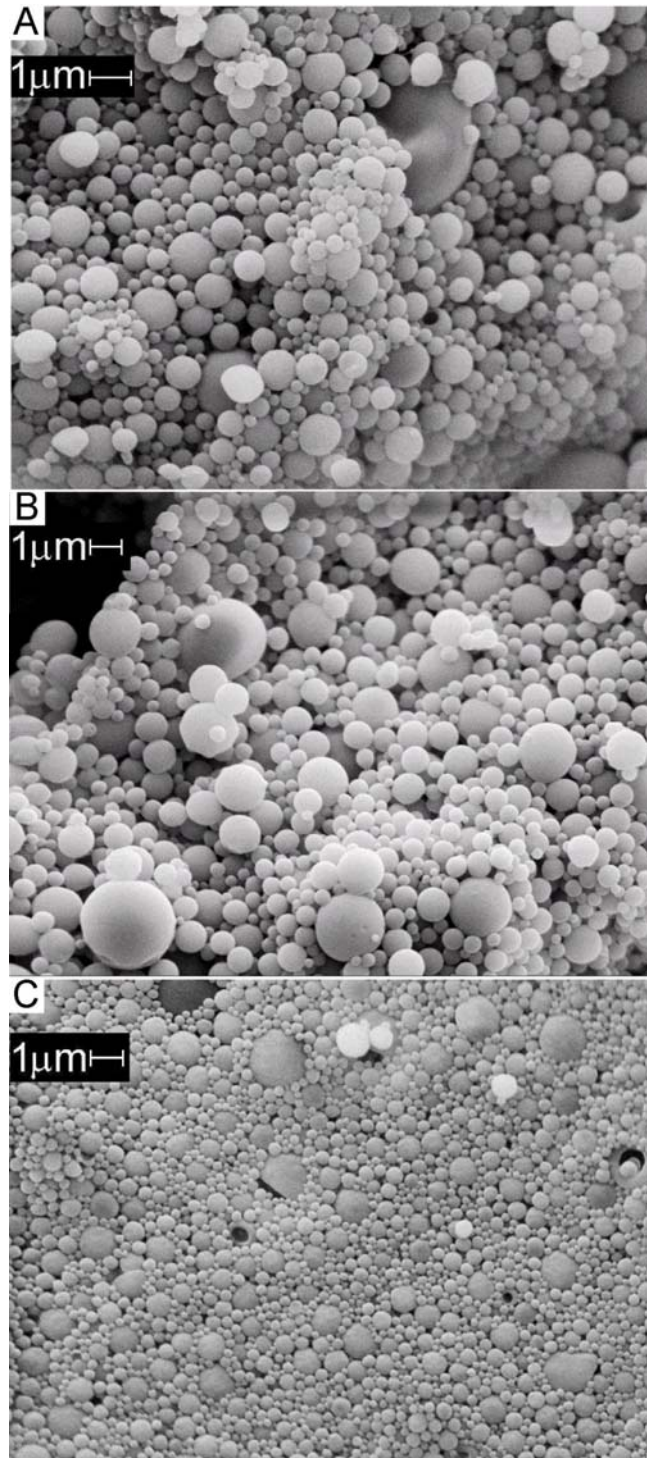


Figure 2.1. Scanning electron micrographs of (A) PLGA/PVA, (B) PLGA/PEMA, and (C) PLGA-COOH/PVA microspheres with no encapsulated protein.

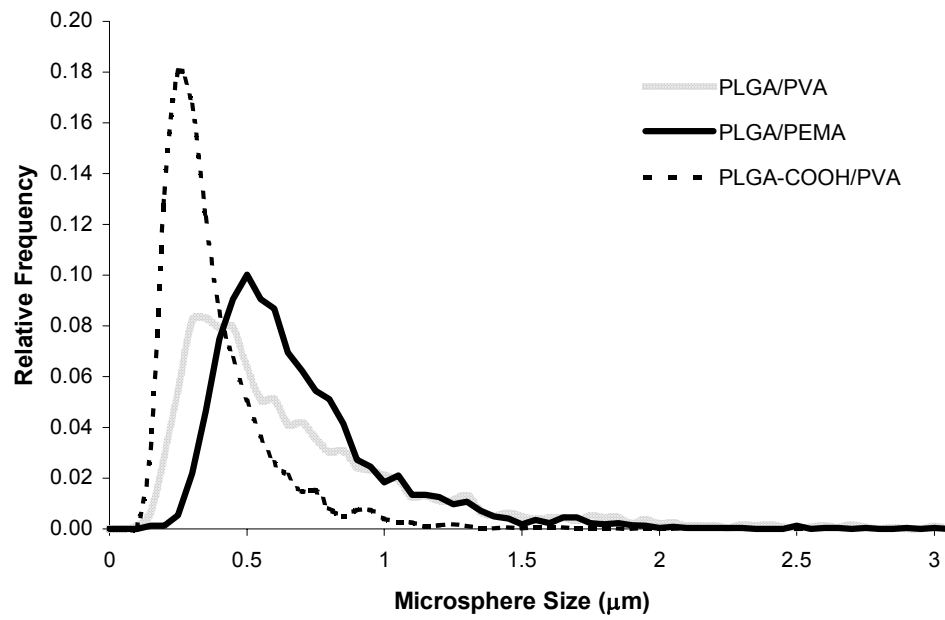


Figure 2.2. Microsphere size distributions (n = 3246 for PLGA/PVA, 2605 for PLGA/PEMA, 3246 for PLGA-COOH/PVA). Microspheres have no encapsulated protein.

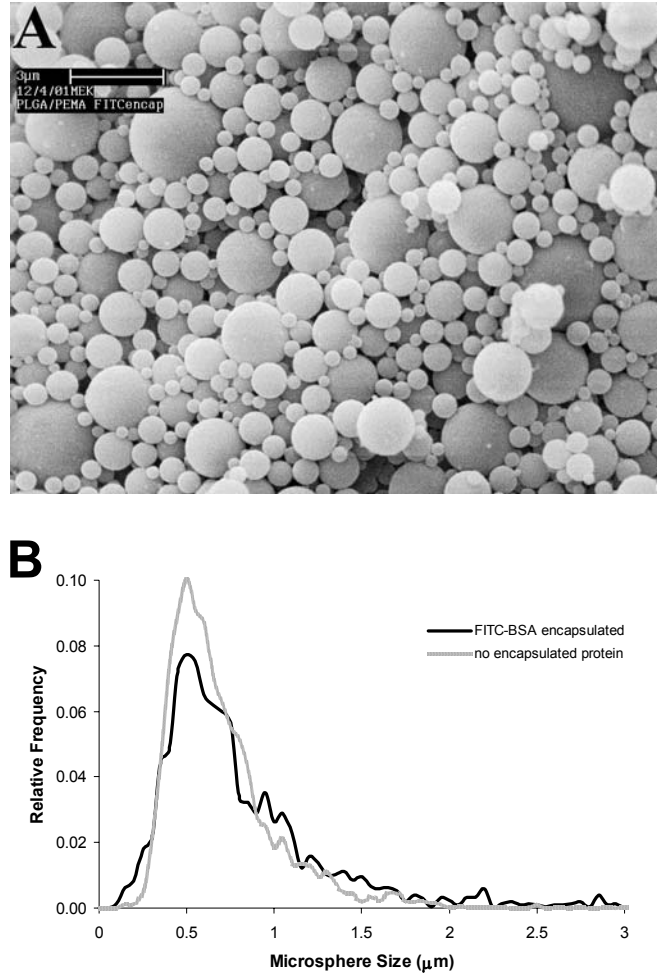


Figure 2.3. PLGA/PEMA microspheres with encapsulated FITC-BSA. A: Microsphere morphology. B: Effect of encapsulated protein on size distribution of PLGA/PEMA microspheres (n = 2083 for FITC-BSA encapsulated microspheres, 2605 for microspheres with no encapsulated protein).

2.3 Conjugation of ligands to microspheres

The technique used to conjugate ligands to the microsphere surfaces is a slightly modified version of a manufacturer's protocol for protein conjugation to carboxylated polystyrene microspheres (Polysciences Technical Data Sheet 238C). Microspheres of PLGA/PVA, PLGA/PEMA, PLGA-COOH/PVA, and carboxylated polystyrene (Polysciences, diameter $0.792 \pm 0.023 \mu\text{m}$) were washed twice with 0.1 M sodium bicarbonate buffer, pH adjusted to 9.0. The microspheres were then washed 3 times with 0.02 M sodium phosphate buffer, pH = 4.8. After washing, the microspheres were resuspended to 20 mg/ml in phosphate buffer and then diluted down to 10 mg/ml with 2% w/v EDC (Sigma) in phosphate buffer. This suspension was incubated for 3 hours at 25°C on an end-to-end shaker to activate carboxylic acid groups. After incubation, microspheres were washed 3 times in phosphate buffer, and resuspended to 10 mg/ml in borate buffer (0.2 M boric acid, pH adjusted to 8.5) with 80 $\mu\text{g/ml}$ of the primary amine-bearing model ligand 5-(aminoacetamido)fluorescein (Molecular Probes, Eugene, OR). Microspheres were incubated with ligand overnight at 25°C on an end-to-end shaker. Microspheres were then centrifuged at 10,000x g for 5 minutes, and the supernatant was removed. The microspheres were resuspended to 10 mg/ml in borate buffer, and 4 μl of 0.25 M ethanolamine in borate buffer was added for each milligram of microspheres. Microspheres were incubated for 30 minutes at 25°C to quench any unreacted sites, and then washed twice with Milli-Q water, resuspended in 4 ml Milli-Q water, and lyophilized to dryness.

The conjugation protocol was also performed with PLGA/PEMA microspheres using the proteins HSA and hIgA (both from Sigma) as ligands, at a concentration of 320 $\mu\text{g/ml}$ during ligand incubation with the microspheres.

The conjugation process did not change microsphere morphology, as shown in the scanning electron micrograph in Figure 2.4. Microsphere surfaces remained smooth and unbroken.

2.4 Detection of conjugated ligands

Conjugation of 5-(aminoacetamido)fluorescein to the surface of PLGA/PEMA microspheres was verified by fluorescence microscopy, using a Zeiss Axiovert 200 inverted microscope (Thornwood, NY) with fluorescent filters. Figure 2.5D shows the presence of the fluorescent ligand on PLGA/PEMA microspheres. Neither adsorption of the ligand to PLGA/PEMA microspheres (Figure 2.3F) or conjugation of the ligand to PLGA/PVA microspheres was detected by microscopy (Figure 2.5B). A more quantitative analysis of 5-(aminoacetamido)fluorescein coupling to the surface of the microspheres was performed by flow cytometry (FACScalibur, Becton Dickinson, San Jose, CA). Fluorescence intensity of PLGA/PEMA microspheres before and after both ligand conjugation and adsorption is shown in Figure 2.6. These dot plots show substantial conjugation of ligand to the microspheres (Figure 2.6B). A slight increase in fluorescence is also observed after adsorption of the fluorescent ligand to the microspheres (Figure 2.6C). Samples of all 4 microsphere formulations with 5-(aminoacetamido)fluorescein conjugated or adsorbed to the surface were analyzed by flow cytometry, and the mean fluorescence intensity was determined for each sample (Figure 2.7). PLGA/PEMA and carboxylated polystyrene microspheres had comparable fluorescence intensities after ligand conjugation. PLGA/PVA and PLGA-COOH/PVA microspheres had only low levels of ligand conjugation, with the PLGA-COOH/PVA microspheres showing slightly more fluorescence (Figure 2.7, inset). This fluorescence is likely due to ligand conjugation to carboxylic acid groups at the ends of PLGA chains. Ligand conjugation is clearly more effective with

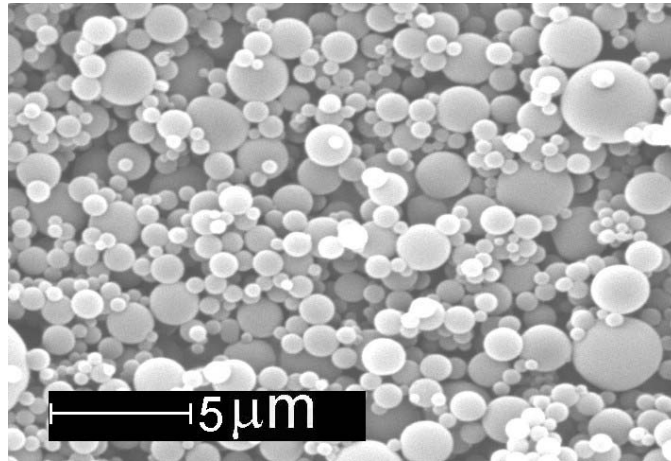


Figure 2.4. Scanning electron micrograph of PLGA/PEMA microspheres after conjugation to 5-(aminoacetamido)fluorescein.

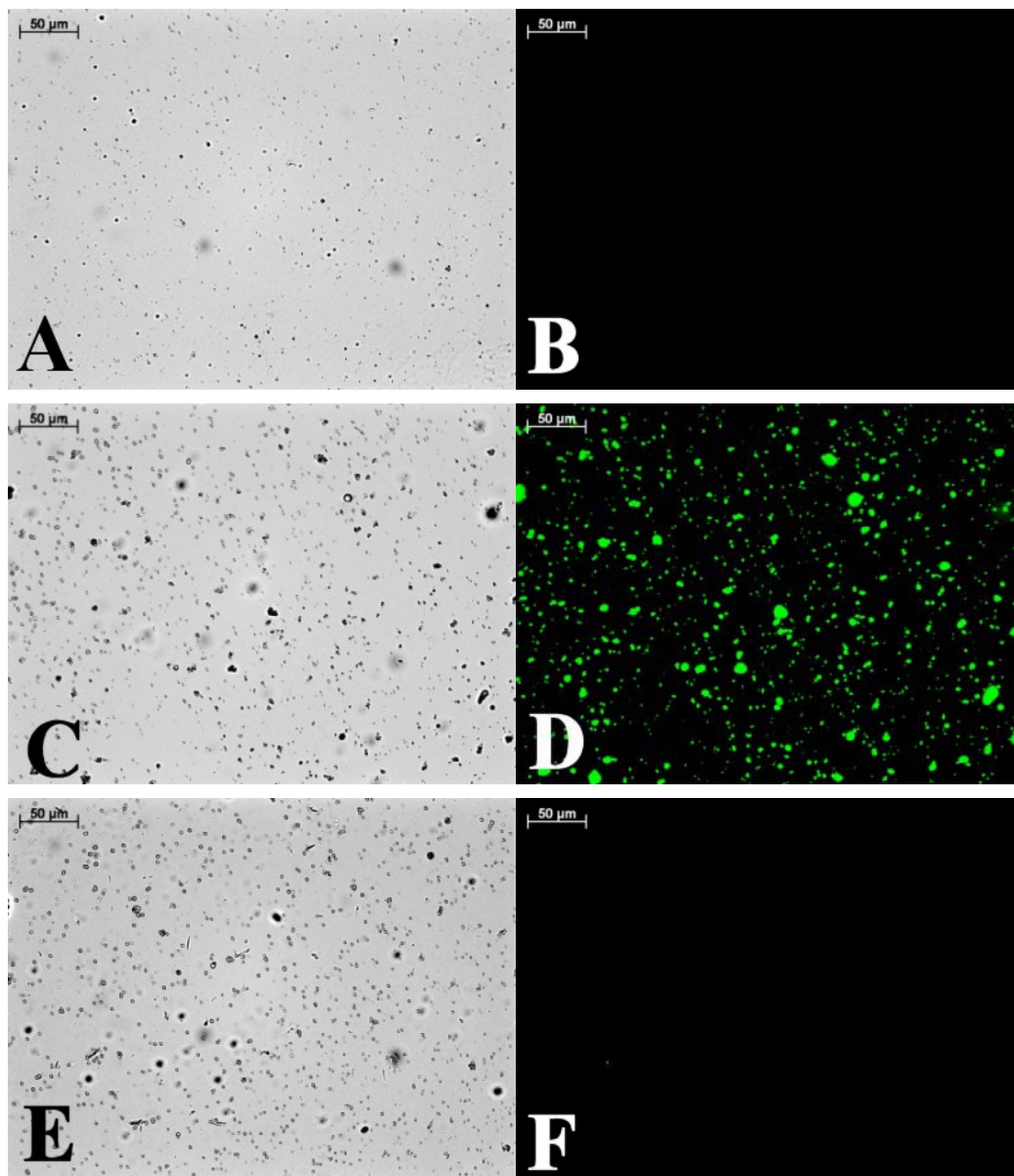


Figure 2.5. Transmission (A, C, E) and fluorescence (B, D, F) micrographs of PLGA microspheres after conjugation to 5-(aminoacetamido)fluorescein. A, B: PLGA/PVA microspheres with conjugated ligand. C, D: PLGA/PEMA microspheres with conjugated ligand. E, F: PLGA/PEMA microspheres with adsorbed ligand.

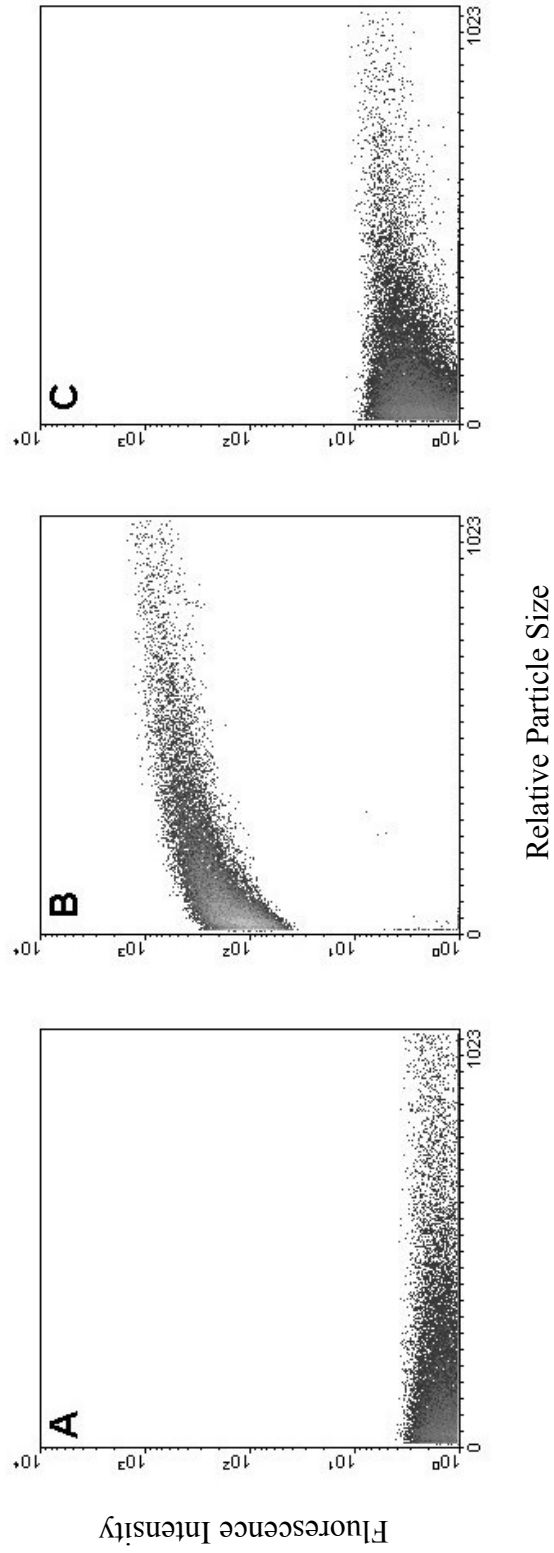


Figure 2.6. Dot plots of fluorescence intensity vs. forward scatter for PLGA/PEMA microspheres. A: Microspheres before conjugation to 5-(aminoacetamido)fluorescein. B: Microspheres after conjugation to ligand. C: Microspheres processed through conjugation protocol without EDC (ligand adsorption)

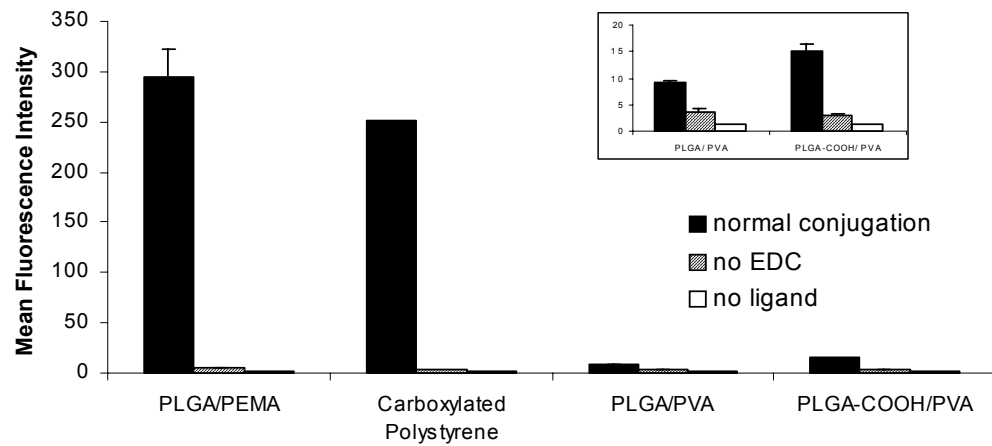


Figure 2.7. Comparison of capacity for coupling of 5-(aminoacetamido)fluorescein to the surface of various microsphere formulations. Data are the mean fluorescence measurement for microspheres as analyzed by flow cytometry. Inset: fluorescence intensity of PLGA/PVA and PLGA-COOH/PVA microspheres. Data are mean \pm standard deviation of mean fluorescence intensity for 3 separate conjugations or adsorptions of ligand to each sphere type.

PLGA/PEMA microspheres, although whether this is because the acid end groups of the PLGA-COOH/PVA microspheres are being shielded by PVA or are simply present in lower numbers than on PLGA/PEMA microspheres remains unclear.

For microspheres with protein conjugated to the surface, aliquots of microspheres were suspended at 5 mg/ml in a 1:500 dilution in PBS of mouse IgG specific for HSA (Sigma) or hIgA (Zymed, South San Francisco, CA). Microspheres were incubated for one hour at room temperature. As a control for non-specific antibody adsorption, separate aliquots of the microspheres were incubated with primary antibody not specific for the conjugated protein. Microspheres were washed 3 times with PBS, and resuspended to 5 mg/ml in a 1:50 dilution in PBS of PE-labeled goat-anti-mouse IgG (Molecular Probes). Microspheres were incubated for one hour at room temperature, and then washed 3 times with PBS before analysis by flow cytometry.

Each of the selected protein ligands could be conjugated to the surface of the PLGA/PEMA microspheres, as shown in Figure 2.8. The presence of the ligand is indicated by the rightward shifts in the fluorescence histograms for microspheres incubated with antibodies specific for the particular ligand (solid fill). Controls for nonspecific primary or secondary antibody adsorption to the microsphere surface (black lines) have nearly equal fluorescence intensity as microspheres not incubated with labeled antibodies at all (gray lines). This suggests that the level of non-specific protein adsorption to the PLGA/PEMA microsphere surface is relatively low in comparison to the amount of protein that can be chemically conjugated via linkages to surface carboxylic acid groups.

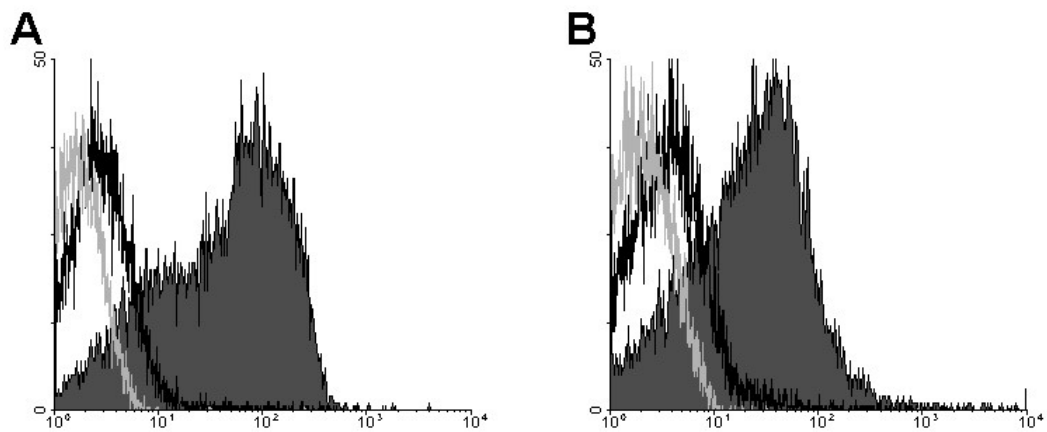


Figure 2.8. Detection by immunocytometry of (A) HSA and (B) hIgA conjugated to PLGA/PEMA microspheres. Histograms are of fluorescence intensity. Gray line: microspheres before incubation with antibodies. Black line: microspheres incubated with irrelevant primary antibody (control for non-specific antibody adsorption). Solid fill: microspheres incubated with primary antibody specific for conjugated protein.

2.5 Encapsulated FITC-BSA release assay

PLGA/PVA and PLGA/PEMA microspheres with encapsulated FITC-BSA were suspended in PBS at a concentration of 2.0 mg/ml and incubated at 37°C on an orbital shaker at 125 rpm. To evaluate the effect of ligand conjugation on encapsulated protein release, PLGA/PEMA microspheres with OVA conjugated to the surface were included in this study. At selected time points the microspheres were centrifuged at 10,000x g for 5 minutes. Supernatant aliquots were removed from each sample, and replaced with an equal volume of fresh PBS. Microspheres were resuspended and returned to the shaker. Supernatant samples were stored at -80°C until analysis. Sample fluorescence was measured on a Perkin Elmer LS 55 luminescence spectrometer (Shelton, CT), with excitation and emission wavelengths set at 488 and 520 nm, respectively.

Changing from PVA to PEMA as the stabilizer during microsphere production did not significantly change the encapsulation efficiency of FITC-BSA (21.4% for PLGA/PVA vs. 24.9% for PLGA/PEMA). Microspheres produced with each surfactant also displayed similar release profiles for encapsulated protein (Figure 2.9). Both sets of microspheres had an initial burst release of encapsulated protein lasting for approximately 2 days, followed by an extended period of slow protein release. PLGA/PEMA microspheres conjugated to protein ligands had lower overall levels of FITC-BSA release, due to loss of encapsulated protein during the ligand conjugation process. In effect, the initial burst release of encapsulated protein for these microspheres took place during ligand conjugation, resulting in microspheres with lower, but still significant, levels of encapsulated protein.

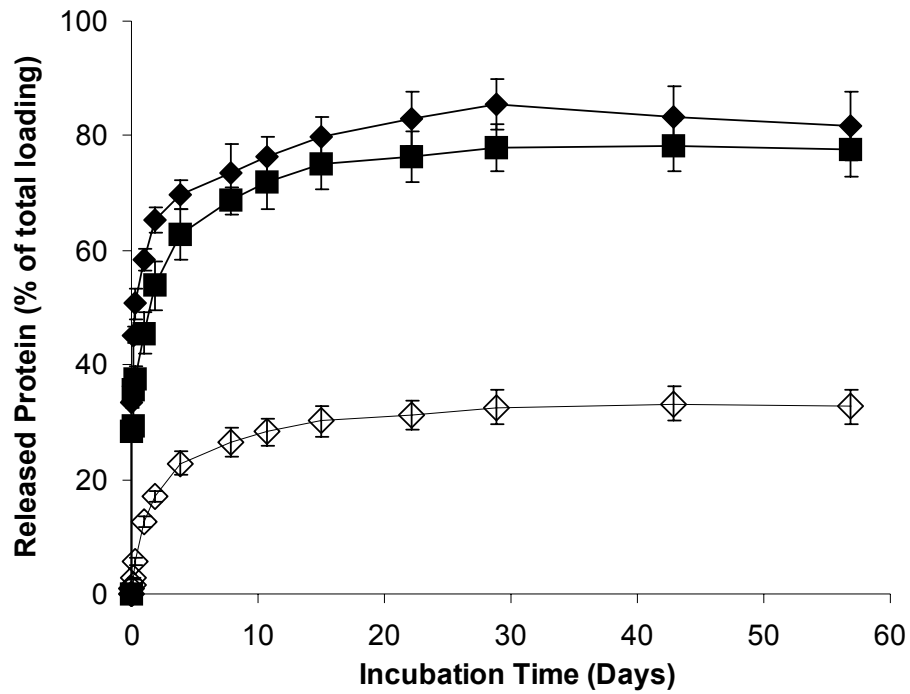


Figure 2.9. Cumulative release of encapsulated FITC-BSA from PLGA microspheres during incubation in PBS at 37°C. Data for PLGA/PEMA microspheres after conjugation protocol (open symbols) represented as percent of original (pre-conjugation) protein loading. ■ = PLGA/PVA microspheres ◆ = PLGA/PEMA microspheres, before conjugation ◇ = PLGA/PEMA microspheres, after conjugation to OVA. Data are means of 3 separate samples for each group, and error bars indicate standard deviation.

2.6 Conjugated ligand release assay

PLGA/PEMA microspheres conjugated to 5-(aminoacetamido)fluorescein were suspended in PBS at 5 mg/ml and incubated at 37°C for various lengths of time. After incubation, microspheres were washed twice with fresh PBS, and resuspended. Retention of fluorescent ligand was measured by flow cytometry of microspheres. Total surface conjugated ligand was estimated by comparison of the fluorescence intensity of microsphere-ligand conjugates to fluorescent standard beads (Quantum™ 26, Flow Cytometry Standards Corp., San Juan, PR).

Fluorescence intensity of PLGA/PEMA microspheres with bound fluorescent ligand was converted to ligand density by comparison to a standard curve relating mean fluorescence to molecules of equivalent soluble fluorochrome (MESF) of polystyrene calibration beads. This comparison indicated that an average of $26,300 \pm 276$ (mean \pm standard deviation of 3 measurements) ligand molecules were conjugated per microsphere. During incubation in PBS at 37°C the microspheres lost approximately 10% of the conjugated ligand within 3 days, at which point ligand release stopped (Figure 2.10). Microsphere fluorescence remained constant from this point to the remainder of the incubation period of 40 days. Supernatants of the incubation buffer were analyzed by fluorometry, and the presence of released ligand in the supernatant was consistent with the release profile of Figure 2.10.

2.7 Summary of ligand conjugation to PLGA/PEMA microspheres

The experiments described in this chapter show that using PEMA as a surfactant to stabilize emulsions during the microsphere production process results in microspheres that have similar properties to microspheres made with PVA as the stabilizer. The PLGA/PEMA microspheres enable ligand conjugation by carbodiimide cross-linking at a level similar to that achieved with carboxylated

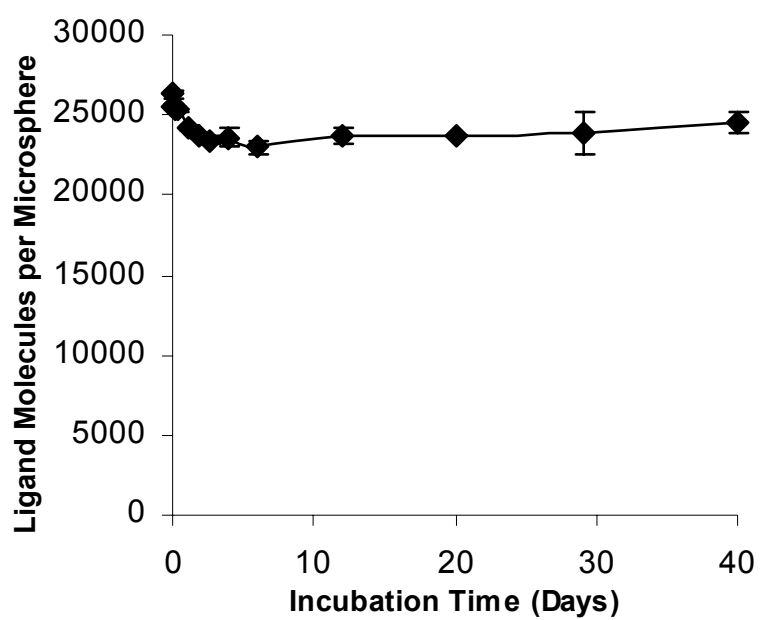


Figure 2.10. Retention of conjugated 5-(aminoacetamido)fluorescein at the surface of PLGA/PEMA microspheres. Data are means of 3 separate samples, and error bars indicate standard deviation.

polystyrene microspheres that are currently used as model particles in applications where microspheres with surface ligands are desired. Even though the PLGA/PEMA microspheres are biodegradable, the linkage between conjugated ligands and the microsphere surface remain stable for extended periods of time, allowing these microspheres to be used for targeting of specific cell types in drug delivery applications.

CHAPTER 3

IN VITRO EVALUATION OF MICROSPHERE TARGETING BY SURFACE- CONJUGATED LIGANDS

3.1 Purpose

The PLGA/PEMA microsphere formulation described in Chapter 2 enables the coupling of ligands to the microsphere surface. A pair of *in vitro* model systems were developed to evaluate the effect of surface-bound ligands in targeting these microspheres to surfaces expressing specific receptors. One model system is a matrix of agarose beads functionalized with receptors. This system presents an environment of narrow pores for the microspheres to travel through, forcing close contact between microsphere-bound ligands and receptors immobilized on the agarose matrix. The other system is a cell culture model, in which monolayers of Caco-2 intestinal epithelial cells are incubated with ligand-conjugated microspheres. In this system, microspheres are floating freely in suspension, meaning that the nature of the microsphere interactions with the targeted surface (in this case the apical surface of the Caco-2 monolayer) is very different from that for the agarose matrix system.

3.2 Retention of targeted microspheres on receptor-bearing agarose matrices

Columns packed with receptor-bearing agarose beads were prepared to investigate the effect of surface ligands on microsphere retention within the columns. A fraction of any microsphere type loaded onto the agarose matrix can be expected to be retained on the column due to simple steric effects. An increase in retention of microspheres due to binding interactions between agarose-bound receptors and microsphere-conjugated ligands, however, would be an effective *in vitro* demonstration that the surface ligand capacity of PLGA/PEMA microspheres is

sufficient to enable the targeting of microspheres to surfaces expressing appropriate receptors.

3.2.1 Conjugation of ligands to PLGA/PEMA microspheres with encapsulated FITC-BSA for targeting to agarose-bound receptors

PLGA/PEMA microspheres with encapsulated FITC-BSA were prepared as described in section 2.1. Conjugation of streptavidin (Sigma), goat antibody specific for mouse IgG (Sigma), and BSA to the microspheres was performed according to the protocol described in section 2.3.

3.2.2 Detection of conjugated ligands on microsphere surfaces

Successful conjugation of the ligands to the microspheres was verified by flow cytometry (Figure 3.1). Detection of goat-anti-mouse IgG and BSA was performed as described in section 2.4, using rabbit-anti-goat IgG (Zymed) and mouse-anti-BSA (Sigma) as primary antibodies, and PE-conjugated goat-rabbit-IgG and goat-anti-mouse IgG (Molecular Probes), respectively, as the secondary antibodies. For microspheres with streptavidin conjugated to the surface, aliquots were suspended at 5 mg/ml in PBS with 5 µg/ml biotin-phycoerythrin conjugate (biotin-PE, Fluka, Buchs, Switzerland). Microspheres were incubated for one hour, washed 3 times with PBS, and then analyzed by flow cytometry. To measure non-specific binding of biotin-PE, control microspheres with BSA conjugated to the surface were also incubated with biotin-PE as described and analyzed by flow cytometry.

Each of the selected protein ligands could be conjugated to the surface of the PLGA/PEMA microspheres, as shown in Figure 3.1. The presence of the ligand is indicated by the rightward shifts in the fluorescence histograms for microspheres incubated with fluorescent labels specific for the particular ligand. For ligands

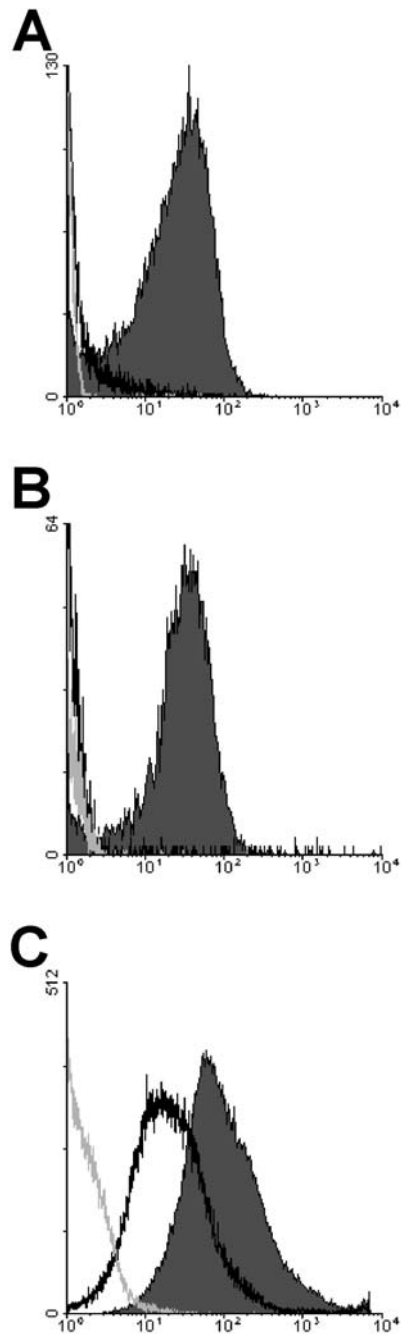


Figure 3.1. Detection of (A) goat-anti-mouse IgG, (B) BSA, and (C) streptavidin conjugated to the surface of PLGA/PEMA microspheres with encapsulated FITC-BSA. Gray line: microspheres before incubation with fluorescent label. Black line: controls for nonspecific binding of fluorescent label to microspheres. Solid fill: microspheres incubated with fluorescent labels specific for conjugated protein.

detected with labeled antibodies (Figure 3.1A, 3.1B), controls for nonspecific antibody adsorption to the microsphere surface (black lines) have nearly equal fluorescence intensity as microspheres not incubated with labeled antibodies at all (gray lines). For microspheres conjugated to streptavidin (Figure 3.1C), the BSA-conjugated control for nonspecific adsorption of the biotin-PE label (black line) show a significant increase in fluorescence intensity compared to unlabeled control (gray line). In this case, however, the fluorescence intensity of the streptavidin-conjugated microspheres is still one logarithm greater than that for the BSA-conjugated control.

3.2.3 Conjugation of mouse IgG to agarose beads

Fifteen ml of a 4% suspension of N-hydroxysuccinimidyl-agarose beads (Sigma) were washed with 30 ml of ice-cold Milli-Q water on a sintered glass filter. The beads were then transferred to a glass bottle and resuspended in 6 ml of a 1 mg/ml solution of mouse IgG (Rockland Immunochemical, Gilbertsville, PA) in 0.05 M sodium bicarbonate buffer (pH = 8.5). The beads were incubated on an end-to-end mixer for 6 hours at 4°C. After this incubation, 3.0 ml of 1 M ethanolamine in bicarbonate buffer was added to the beads, which were then incubated for 1 hour to quench any remaining unreacted sites on the beads. The mouse IgG-conjugated agarose beads were then washed 5 times with cold PBS (15ml/wash), and then stored in PBS as a 4% suspension at 4°C until use.

3.2.4 Retention of Microspheres on Agarose Columns

1.5 ml of a 4% suspension of either agarose-biotin (Sigma) or mouse IgG-agarose was added to glass columns (1 cm diameter) and allowed to settle overnight. After washing the column with 2 ml PBS, 1 ml of a 5 mg/ml suspension of microspheres conjugated to either BSA, streptavidin, or goat antibody specific for

mouse IgG was added to the top of the agarose bed, followed by 20 ml PBS. After the PBS was eluted, 15 ml of 6 M guanidine HCl (Sigma) pH adjusted to 7.5 was run through the column. Eluate was collected in 1ml fractions, and the fluorescence intensity of each fraction was measured by fluorometry with excitation and emission wavelengths of 488 and 520 nm, respectively. Fluorescence intensity of each fraction was converted to microsphere concentration by comparison to standard curves generated with suspensions of microspheres of each microsphere-ligand combination in both PBS and 6 M guanidine HCl.

When ligand-conjugated microspheres were eluted from agarose columns, a significant quantity of the loaded microspheres were retained on the column, regardless of the particular microsphere ligand or agarose-bound receptor (Figures 3.2 and 3.3). The highest fraction of microspheres recovered in the eluate for any of the trials was approximately 55%. The fraction of microspheres retained increases markedly, however, when the microsphere ligand is matched to the appropriate agarose-bound receptor molecule. For the streptavidin-conjugated microspheres, total retention was roughly 50% on the agarose-mouse IgG column, but increased to 95% when the microspheres were introduced onto an agarose-biotin column (Figure 3.2). Microspheres with BSA conjugated to the surface were retained at a level of approximately 55% on the agarose-biotin matrix, indicating that the high level of retention for streptavidin-conjugated microspheres on the agarose-biotin column is due to specific interaction of microsphere-bound streptavidin with biotin coupled to the agarose matrix. Similar results were obtained with microspheres conjugated to goat antibody specific for mouse IgG (Figure 3.3). On the agarose-biotin matrix, approximately 80% of the microspheres were retained on the column, whereas nearly 95% were retained on the agarose-mouse IgG column.

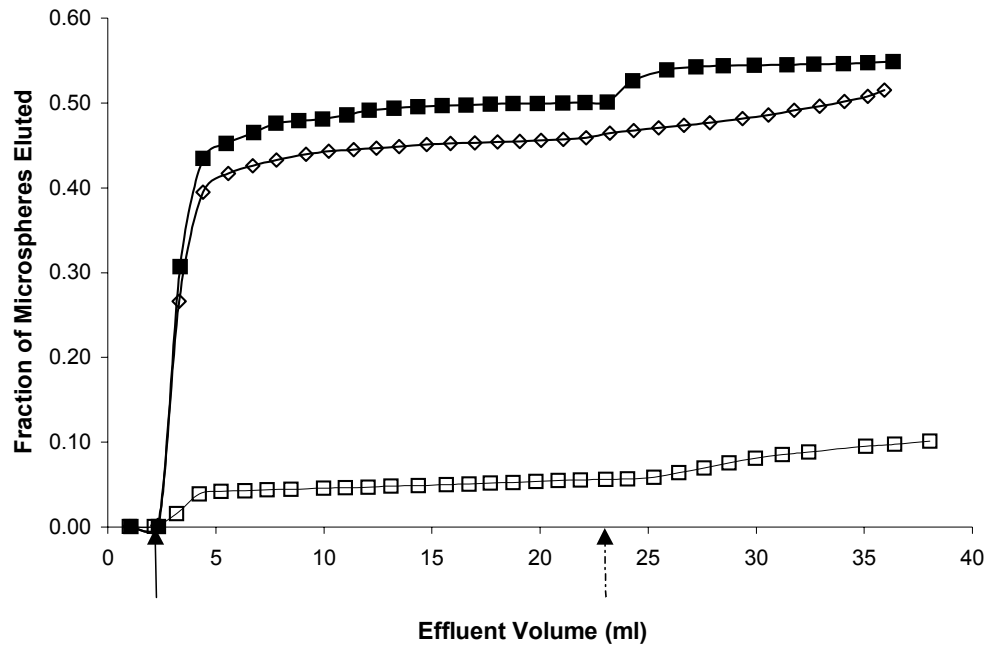


Figure 3.2. Retention of streptavidin-conjugated microspheres on agarose columns. □ = streptavidin-conjugated microspheres, agarose-biotin column. ■ = streptavidin-conjugated microspheres, agarose-mouse IgG column. ◇ = BSA-conjugated microspheres, agarose-biotin column. Solid arrow: addition of microspheres to column. Dotted arrow: switch in elution buffer to 6M guanidine HCl.

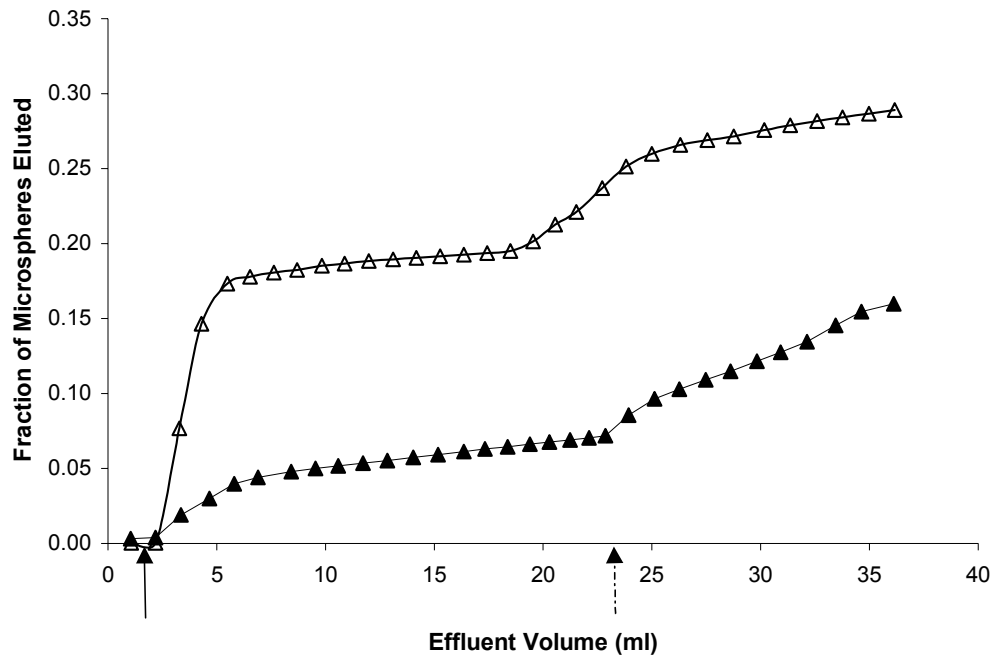


Figure 3.3. Retention of goat-anti-mouse IgG-conjugated microspheres on agarose columns. \triangle = agarose-biotin column. \blacktriangle = agarose-mouse IgG column. Solid arrow: addition of microspheres to column. Dotted arrow: switch in elution buffer to 6M guanidine HCl.

Changing buffer from PBS to 6 M guanidine HCl during elution did not result in release of additional BSA- or streptavidin-conjugated microspheres from either the agarose-biotin or agarose-mouse IgG columns (Figure 3.2). For the goat antibody-conjugated microspheres, the change in buffer was followed by elution of approximately 10% of the microspheres from the agarose-mouse IgG column (Figure 3.3). These microspheres showed a similar two-step elution profile on the agarose-biotin column, but the increase in elution rate started before the switch of elution buffer to 6M guanidine HCl, suggesting that it was caused by factors other than the change in elution buffer.

3.3 Adhesion of targeted microspheres to Caco-2 cell monolayers

To model microsphere targeting to epithelial cells, monolayers of Caco-2 intestinal epithelial cells were used. The lectin UEA 1 binds to the apical surface of Caco-2 cell monolayers, and is used for labeling the apical surface of the monolayers [59], indicating that it is a suitable ligand for evaluating the ability of microsphere-ligand conjugates to target surface receptors on monolayers of Caco-2 cells. UEA 1 binds to carbohydrates with terminal fucose residues, allowing the UEA 1 binding interaction with the cell surface to be inhibited through the addition of L-fucose to the incubation medium. If microsphere targeting is being mediated by the intended ligand/receptor binding interaction, co-administration of the inhibitor with microspheres should negate targeting, so that the microsphere interaction with the targeted surface will be equivalent to that of control untargeted microspheres.

3.3.1 Preparation of PLGA/PEMA microspheres for monolayer targeting

Conjugation of the lectin UEA 1 (Vector Laboratories, Burlingame, CA) to PLGA/PEMA microspheres with encapsulated FITC-BSA was performed according

to the protocol described in section 2.3. Preparation of control microspheres with encapsulated FITC-BSA and conjugated BSA was described in section 3.2.1. Successful conjugation of UEA 1 to the microspheres was verified by immunocytometry (Figure 3.4), performed as described in section 2.4, using rabbit-anti-UEA 1 (Sigma) as the primary antibody and PE-conjugated goat-anti-rabbit IgG as the secondary antibody. For the control sample of UEA 1 conjugated microspheres incubated with nonspecific antibody (Figure 3.4, black line), a slight increase in fluorescence is seen as compared to the other samples incubated with antibody nonspecific for the conjugated ligand (Figures 3.1A, 3.1B). The nonspecific control antibody used with the UEA 1-conjugated microspheres was specific for BSA, making it likely that the observed slight increase in fluorescence is due to anti-BSA antibody binding to FITC-BSA entrapped near the surface of the microspheres. This control is significant with regard to the BSA-conjugated microspheres (Figure 3.1B) as well, because it demonstrates that the level of anti-BSA antibody binding to FITC-BSA is relatively low. The substantial rightward shift in fluorescence seen in Figure 3.1B is therefore due to antibody binding to surface-conjugated BSA, and not to entrapped FITC-BSA.

3.3.2 Caco-2 cell culture

The method for growing Caco-2 cells on porous filters has been described elsewhere[60,61]. Caco-2 intestinal epithelial cells of subclone BBel (ATCC, Rockville, MD) were propagated in 75 cm² flasks. The cells were grown in DMEM (4.5 g/l glucose, 10 mM HEPES), supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% nonessential amino acids, and 40 µg/ml human transferrin (all from Gibco, Grand Island, NY). Cells were split 1/8 when they reached confluency (typically 6-7 days), and the medium was changed every 2 or 3

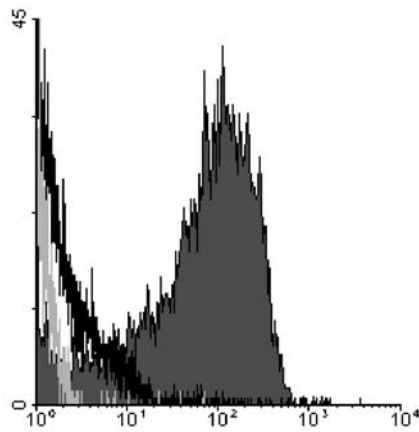


Figure 3.4. Detection of UEA 1 conjugated to the surface of PLGA/PEMA microspheres with encapsulated FITC-BSA. Gray line: microspheres before incubation with fluorescent label. Black line: controls for nonspecific binding of fluorescent label to microspheres. Solid fill: microspheres incubated with fluorescent labels specific for conjugated protein.

days. Polycarbonate Transwell™ filters (6.5 mm diameter, 3 µm pore size) (Corning Costar, Acton, MA) were inverted and placed in open Petri dishes. The filters were coated with bovine collagen (Vitrogen®, Collagen Corporation, Palo Alto, CA), 15 µl per filter of a 2.8 mg/ml solution. The filters were left in a sterile hood overnight to dry, and then placed in PBS until use. The collagen-coated filters were inverted in Petri dishes and seeded with 100 µl media containing 2.5×10^5 cells. The Petri dishes were covered and incubated overnight at 37°C in a 5% CO₂ incubator to allow cell attachment. Filters were then returned to normal orientation in 24-well plates. 500 µl media was added to the lower (apical) chamber, and 125 µl media added to the upper (basolateral) chamber. Medium was changed every two days, and cell monolayers were grown for 21 days before use in microsphere adhesion experiments. Figure 3.5 summarizes the process for seeding Caco-2 cells to the filters.

Transepithelial electrical resistance of the Caco-2 monolayers was measured periodically with an EVOM™ voltmeter/ohmmeter (World Precision Instruments, Sarasota, FL). For consistency, measurements were always taken immediately after the media in both chambers was replaced. Resistance measurements indicated that Caco-2 cells grown on filters formed differentiated monolayers within 3 weeks after seeding. Transepithelial electrical resistance reached approximately 400 Ohm·cm² in this period (Figure 3.6), which is consistent with other reports for the resistance of well-differentiated monolayers of Caco-2 cells [60].

3.3.3 Microsphere Adhesion Study

Microspheres with surface-conjugated BSA or UEA 1 were suspended in Caco-2 medium at 1 mg/ml. L-fucose (Sigma) was added to an aliquot of the UEA 1-conjugated microsphere suspension to a concentration of 500 mM, to inhibit lectin-specific binding of microspheres to the Caco-2 monolayers. 600 µl aliquots of

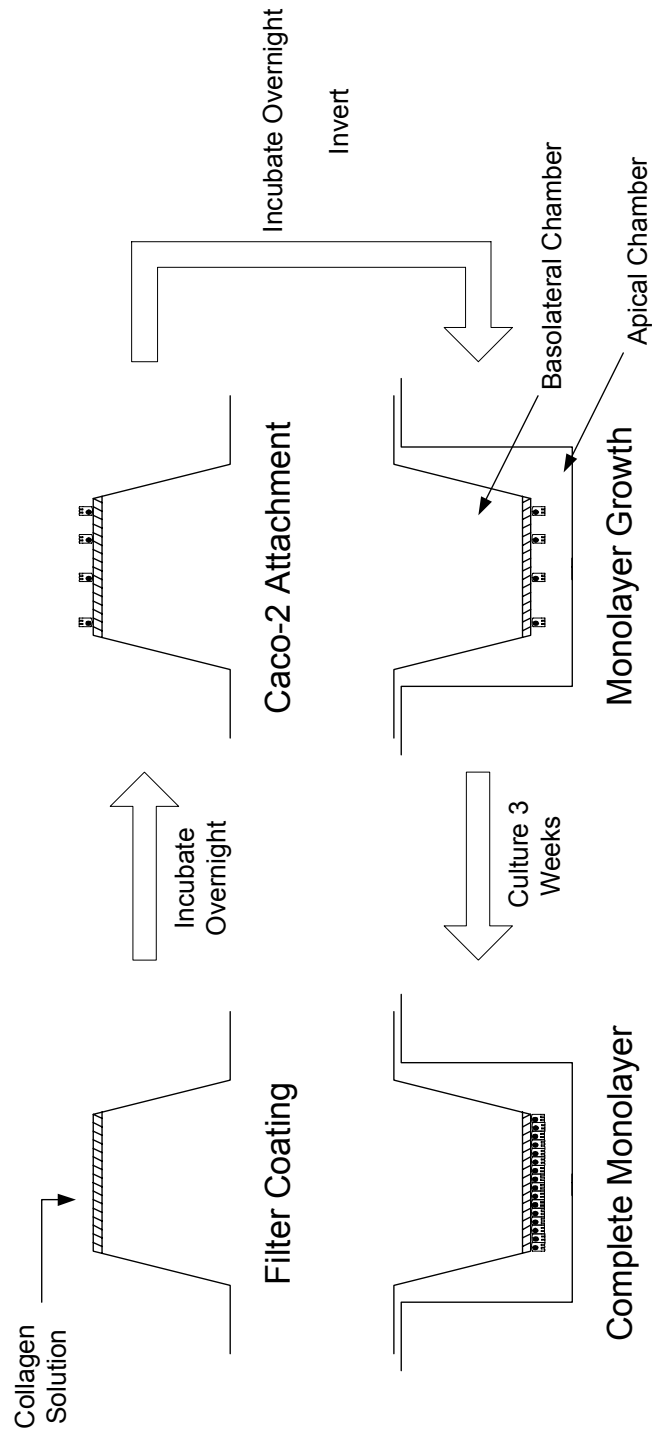


Figure 3.5. Caco-2 cell culture on Transwell™ filters. Filters are coated with collagen to promote cell attachment. After seeding, cells are incubated on inverted filters overnight. Filters are then returned to normal orientation, and cells are grown for 3 weeks to form complete, unbroken monolayers.

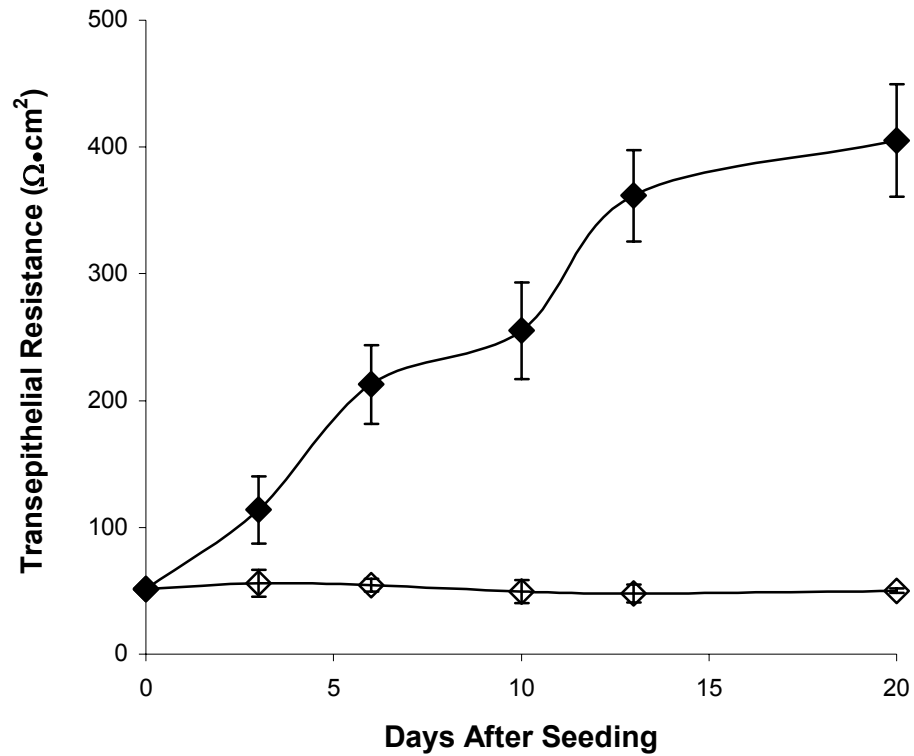


Figure 3.6. Trans epithelial electrical resistance measurements for Caco-2 cells grown on Transwell™ filters. ◆ = Filters with cells. ◇ = Unseeded filters. Data are means ± standard deviations of individual measurements on filters at each time point (n = 4 filters for unseeded filters, n = 12 for seeded filters).

the microsphere suspensions were added to 24-well plates. Caco-2 monolayers on filters were placed in the wells, and 125 μ l of fresh media was added to the basolateral chambers. Plates were covered and incubated at 37°C in 5% CO₂ for 1 hr. After incubation, filters were transferred to new 24-well plates, and media in the basolateral chamber was removed. Filters were moved to wells containing 500 μ l 2% paraformaldehyde in PBS, and 125 μ l of paraformaldehyde solution was added to basolateral chambers. Filters were incubated in this fixative for 30 min, and then transferred to wells containing PBS. Filters were cut out of their supports with a scalpel, and mounted on microscope slides in Vectashield with DAPI (Vector Laboratories) and sealed with a glass cover slip and clear nail polish.

3.3.4 Microsphere Counting

Cell monolayers were viewed with a Zeiss Axiovert 200 inverted microscope with fluorescent filters. Monolayers were viewed with a 20X objective, and 5 digital images were captured for each filter. Sample images are shown in Figure 3.7. Microspheres per unit area were determined for each filter by visual counting of microspheres in each image. Monolayer filter order was randomized for both the image capture and microsphere counting steps, which were performed by operators unaware of which microsphere type each individual filter was incubated with.

BSA-conjugated microspheres adhered to the Caco-2 monolayers at a density of 710 ± 205 microspheres/mm² (mean \pm SD for 3 filters) (Figure 3.8). Microspheres with UEA 1 as the surface-bound ligand showed a two-fold increase in adherence density, at 1330 ± 118 microspheres/mm². Incubating the UEA 1-conjugated microspheres with monolayers in medium containing 500 mM L-fucose inhibitor negated the targeting effect of the ligand, as the adhesion density was reduced to 690 ± 78 microspheres/mm². This adhesion density is essentially equal to that of the BSA-

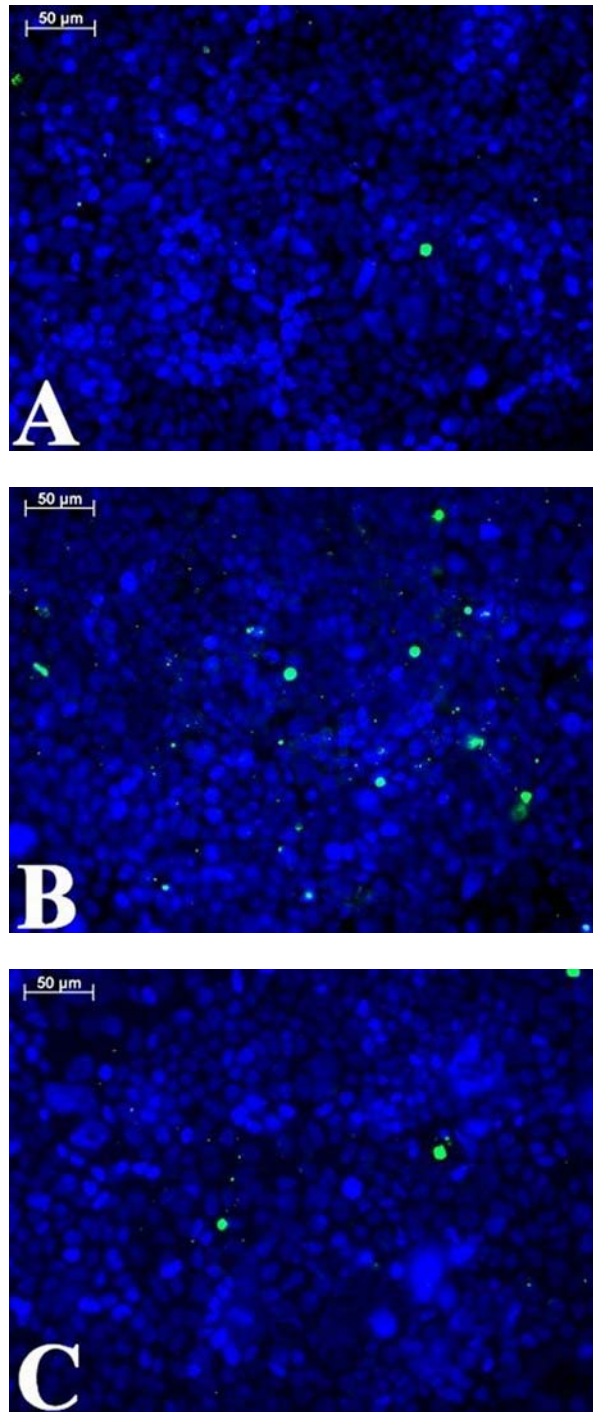


Figure 3.7. PLGA/PEMA microspheres (green) adhering to the apical surface of Caco-2 cell monolayers. Cell nuclei are stained with DAPI (blue). A: BSA-conjugated microspheres. B: UEA 1-conjugated microspheres. C: UEA 1-conjugated microspheres with 500 mM L-fucose.

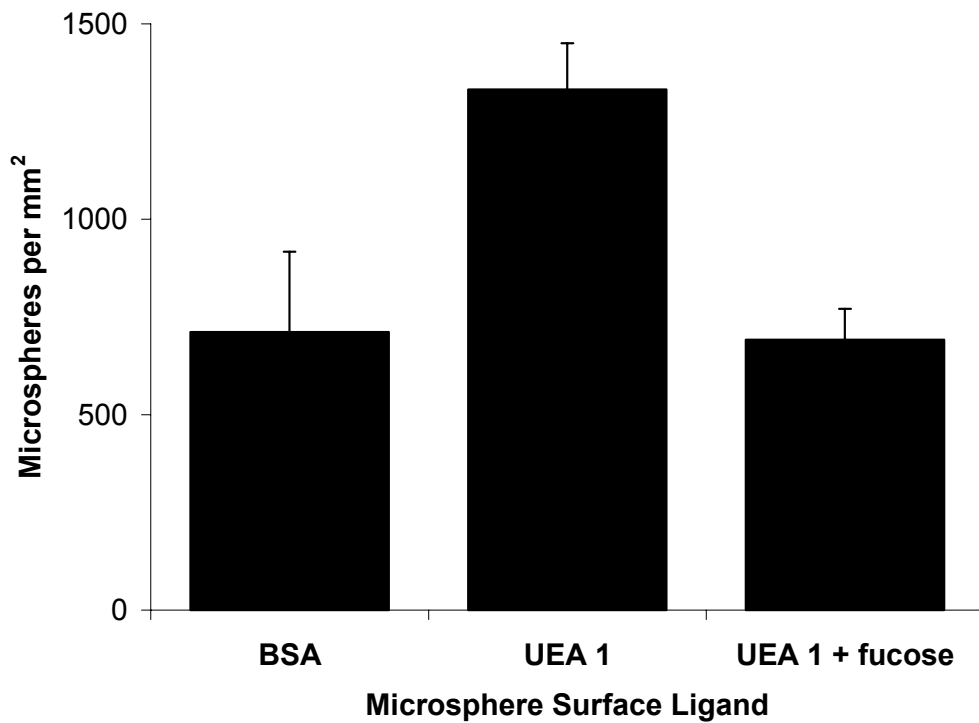


Figure 3.8. Effect of surface ligands on microsphere adhesion to Caco-2 monolayers. Data are means \pm standard deviation for individual filters (3 filters per group, 5 images per filter). $p = 0.018$ for two-tailed Student's t test comparison of results for BSA and UEA 1-conjugated microspheres.

conjugated microspheres, indicating that the observed increase in adhesion density for UEA 1-conjugated microspheres is due to specific interactions of the UEA 1 with receptors on the apical surface of the epithelial cell monolayers. Repetition of the adhesion experiment with fresh Caco-2 monolayers gave similar results, in which the presence of UEA 1 on the microsphere surface doubled the adhesion density of the microspheres compared to microspheres with surface-conjugated BSA.

3.4 Conversion of Caco-2 monolayers to M cells

Efforts to isolate and immortalize M cells have proven unsuccessful to date. Such a cell line would be of great utility for *in vitro* investigation of the mechanisms of pathogen invasion across the intestinal epithelium, as well as for the testing of potential oral vaccine formulations. M cell isolation is a daunting task in part because M cells are present in such low numbers in the epithelium, and also because there is evidence that M cells are not truly a distinct cell type, but are simply regular epithelial cells expressing a different phenotype in response to factors in their immediate external environment [21,62,63]. In light of this, Kerneis and coworkers have developed an M cell model using monolayers of Caco-2 cells cocultured with mouse Peyer's patch lymphocytes [59]. Caco-2 cells in this model display several features of M cells, including poorly organized microvilli at the apical brush border and the ability to transcytose microparticles. In this model, lymphocytes migrate through the pores of the filter the Caco-2 cells are attached to in order to come into direct contact with the Caco-2 cells [59]. Caco-2 cell conversion can be achieved by coculture with mouse Peyer's patch lymphocytes or cells of the human Raji B cell line, but not human Jurkat T cells [59]. This coculture model has been used by a number of groups [59,61,64-66], who report similar findings with regard to microparticle uptake. This model provides an opportunity to evaluate potential targeting ligands for human M

cells such as hIgA [67] and SLAA [40] for their ability to increase microsphere uptake.

3.4.1 Coculture of Caco-2 monolayers with Raji B cells

Caco-2 cell monolayers were grown as described in section 3.3.2. Raji B cells (ATCC) were cultured in T-175 flasks at 37°C in 5% CO₂, suspended in RPMI 1640 medium (ATCC) supplemented with 10% FBS. For coculturing, Raji cells were suspended in DMEM identical to that described in section 3.3.2, except with a glucose concentration of 1.0 g/l (low glucose DMEM) [61]. Raji cells were suspended at densities of 2x10⁶ and 2x10⁷ cells/ml. Caco-2 monolayers grown on filters for 20 days were transferred to wells containing 500 µl low glucose DMEM, and 100 µl of the Raji cell suspension was added to the basolateral chambers to make cocultures with either 2x10⁵ or 2x10⁶ Raji cells/filter. Control monolayers received 100 µl low glucose DMEM containing no Raji cells. Cocultures were incubated for 2 days at 37°C in 5% CO₂ to allow for M cell conversion.

3.4.2 Staining of monolayers

Two days after seeding the filters with Raji B cells, filters were removed from media and transferred to wash wells containing 500 µl cold PBS. 125 µl cold PBS was added to the basolateral chamber. PBS was removed, and filters were transferred to fresh wells containing 500 µl 2% paraformaldehyde in PBS. 125 µl of the paraformaldehyde solution was added to the basolateral chamber. Filters were fixed in the paraformaldehyde/PBS solution for 30 minutes, and then washed twice with fresh PBS as described above. Filters were then transferred to wells containing 500 µl/well 0.3% saponin (Sigma) and 0.1% BSA in PBS (permeabilization buffer). 125 µl permeabilization buffer was added to the basolateral chamber, and filters were

incubated for 30 minutes. Filters were then incubated for 15 minutes in 50 mM NH_4Cl in PBS, and then washed once with PBS and once with permeabilization buffer. The filters were then incubated in 16% goat serum (Gibco), 0.3% saponin in PBS (blocking buffer) for 30 minutes. After blocking, the filters were excised from their supports with a razor blade and placed on microscope slides (monolayer facing up). Filters were then incubated overnight in 25 μl blocking buffer containing 40 $\mu\text{g/ml}$ rhodamine-conjugated UEA 1 (Vector Laboratories) and either 20 $\mu\text{g/ml}$ hIgA or 4 $\mu\text{g/ml}$ mouse monoclonal anti-SLAA (clone KM231, Kamiya Biomedical, Seattle, WA). After overnight incubation, the filters were washed 3 times with permeabilization buffer, and then incubated for 45 minutes in 25 $\mu\text{l}/\text{filter}$ blocking buffer containing 1:25 diluted FITC-goat-anti-human IgA (hIgA stained filters) or FITC-goat-anti-mouse IgG (anti-SLAA stained filters). Filters were then washed 3 times with permeabilization buffer, mounted with Vectashield containing DAPI, and sealed with a glass cover slip and clear nail polish.

Stained filters were viewed on a Zeiss Axiovert 200 inverted microscope with fluorescent filters. Cells in the Caco-2 monolayers stained unevenly with rhodamine-UEA 1, with some cells brightly stained while others showed little if any rhodamine-UEA 1 binding at all. Monolayers not cocultured with Raji B cells did not bind hIgA (Figure 3.9). SLAA expression showed an interesting pattern, with a small fraction of the cells staining brightly (Figures 3.10B and 3.11A). Cells staining brightly for SLAA did not typically stain with rhodamine-UEA 1. Coculture with 2×10^5 Raji cells/filter did not change the apical surface expression of SLAA (Figure 3.13B) or receptors for UEA 1 (Figures 3.12C and 3.13C) and hIgA (Figure 3.12B). The absence of a change in Caco-2 apical expression of hIgA receptors or SLAA in response to coculture with Raji cells indicates that targeting these receptors would not increase microsphere uptake in this M cell model. Coculture with 2×10^6 Raji

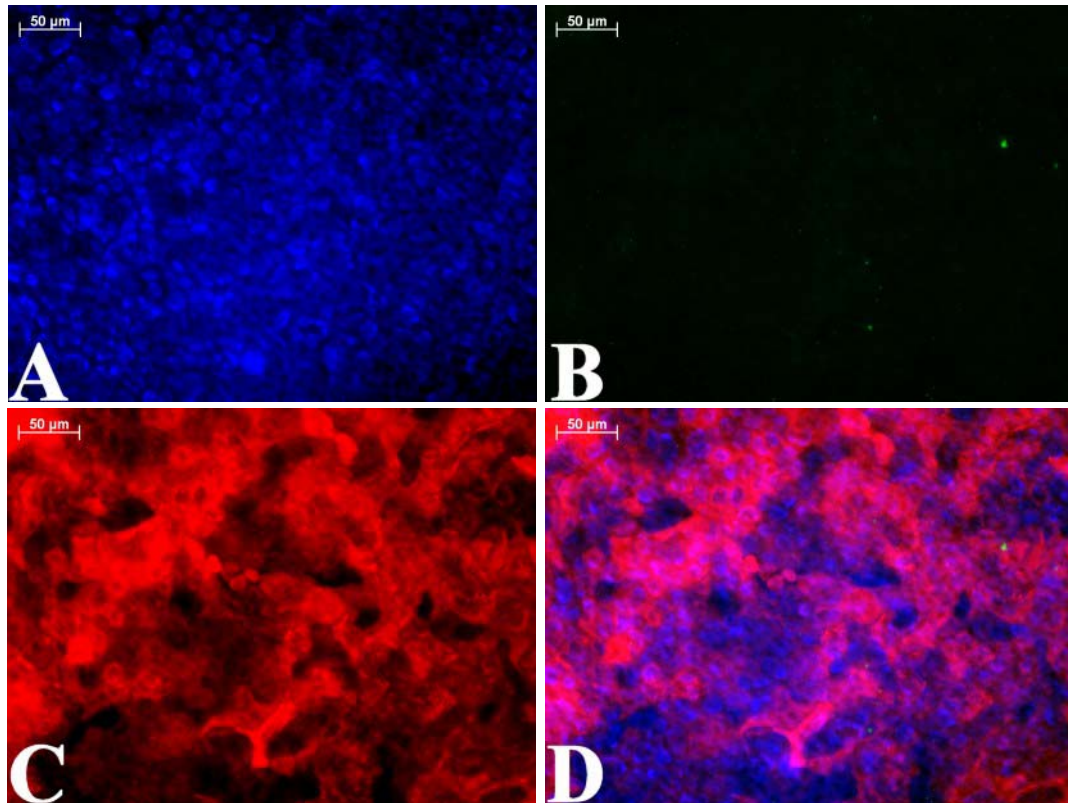


Figure 3.9. hIgA receptor expression on Caco-2 monolayers 21 days after culture. A: Staining of cell nuclei with DAPI. B: Staining for hIgA receptors with FITC. C: Staining of Caco-2 apical surface with rhodamine-UEA 1. D: Composite of figures A-C.

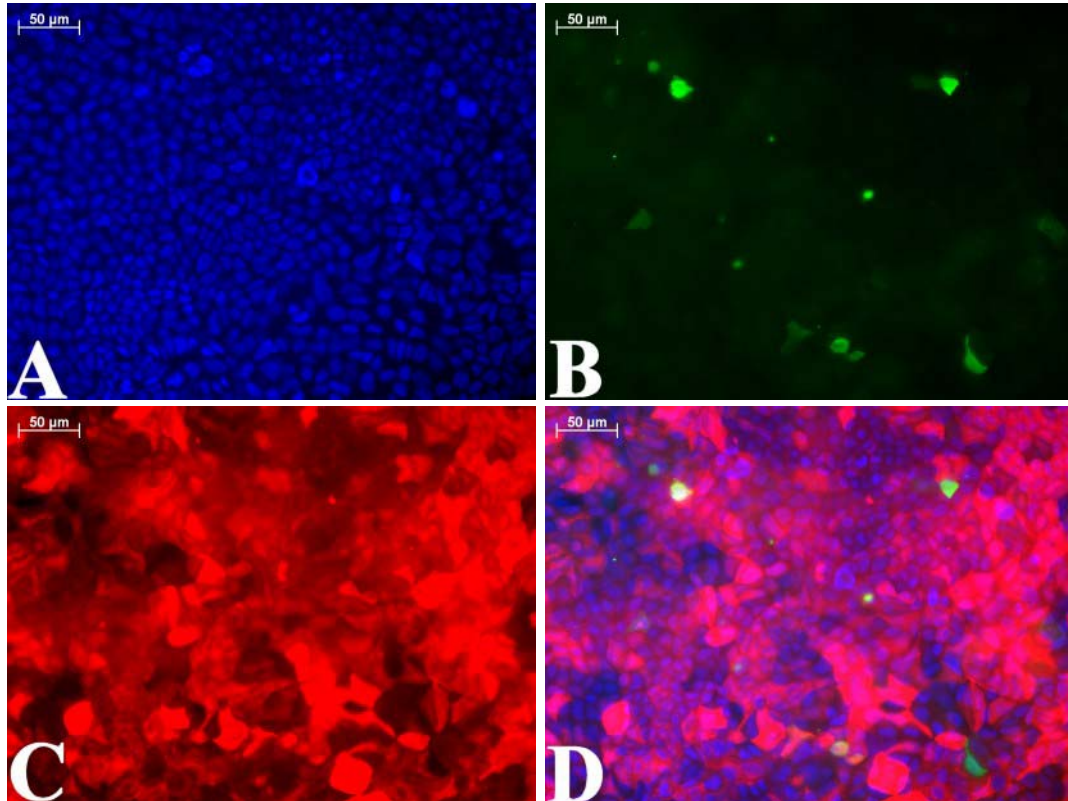


Figure 3.10. Sialyl Lewis A antigen expression on Caco-2 monolayers 21 days after culture. A: Staining of cell nuclei with DAPI. B: Staining for sialyl Lewis A antigen with FITC. C: Staining of Caco-2 apical surface with rhodamine-UEA 1. D: Composite of figures A-C.

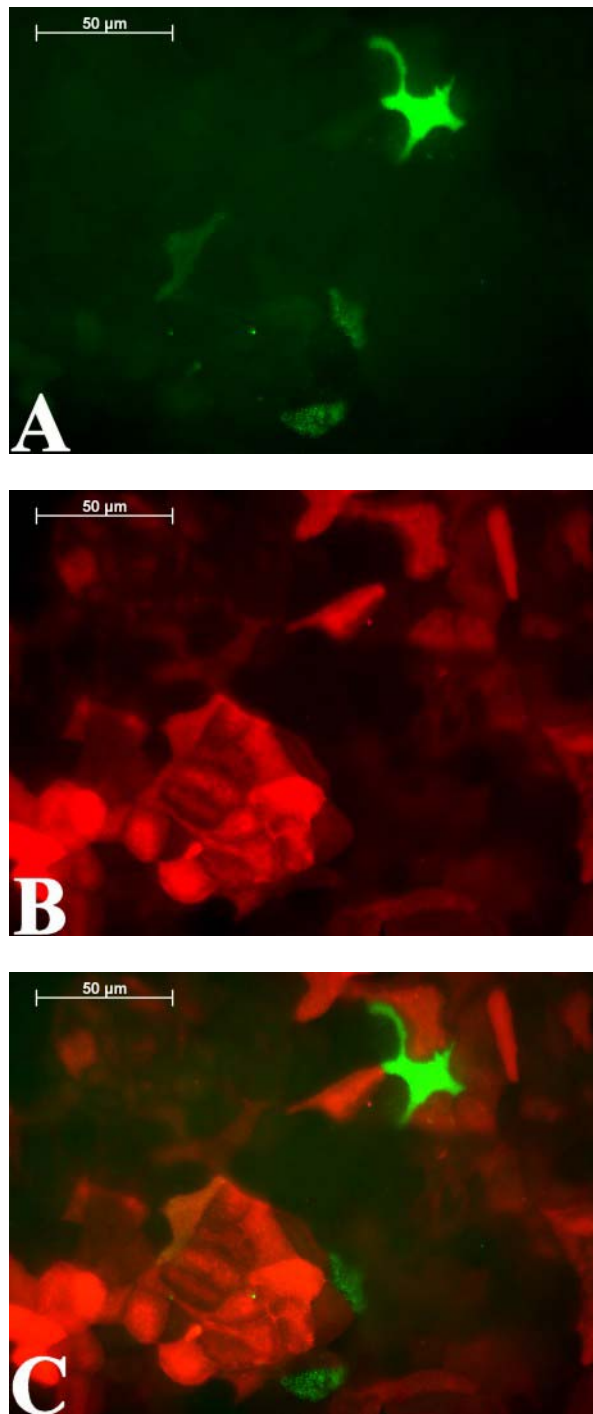


Figure 3.11. Selective expression of sialyl Lewis A antigen on Caco-2 cell apical surfaces. A: Staining for sialyl Lewis A with FITC. B: Rhodamine-UEA 1 staining of Caco-2 apical surface. C: Composite of A and B. Most cells expressing sialyl Lewis A do not bind UEA 1.

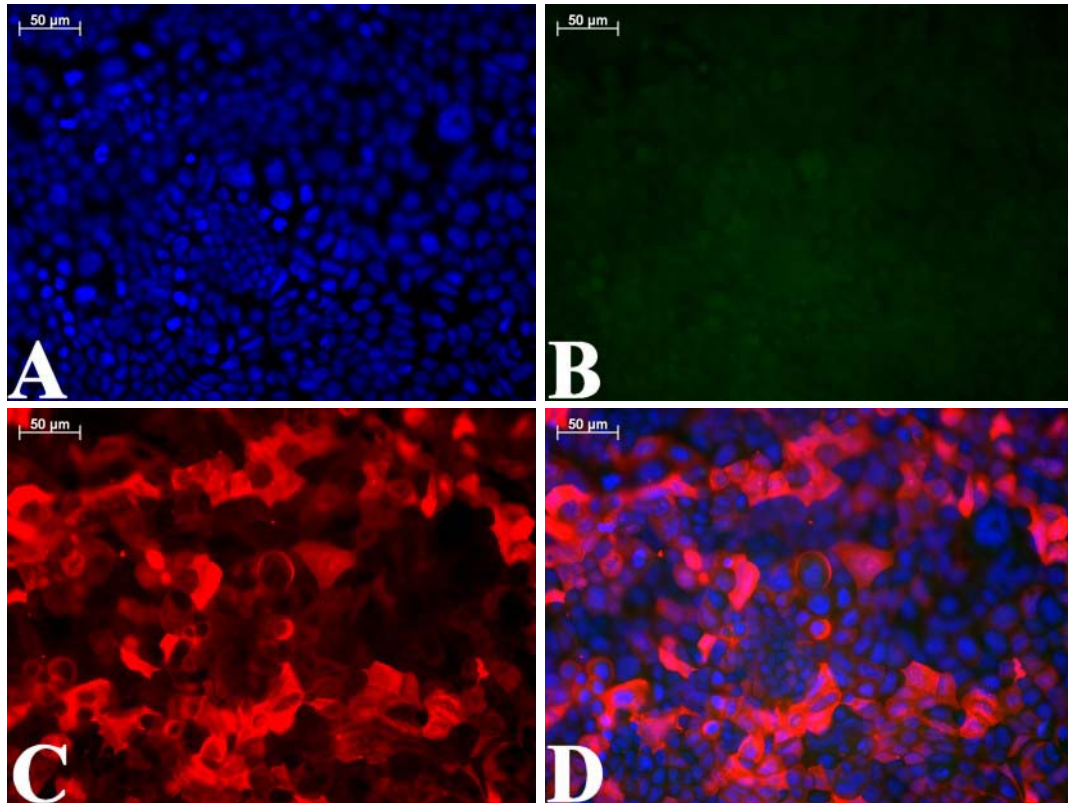


Figure 3.12. hIgA receptor expression on Caco-2 monolayers after coculture with 2×10^5 Raji B cells. A: Staining of cell nuclei with DAPI. B: Staining for hIgA receptors with FITC. C: Staining of Caco-2 apical surface with rhodamine-UEA 1. D: Composite of figures A-C.

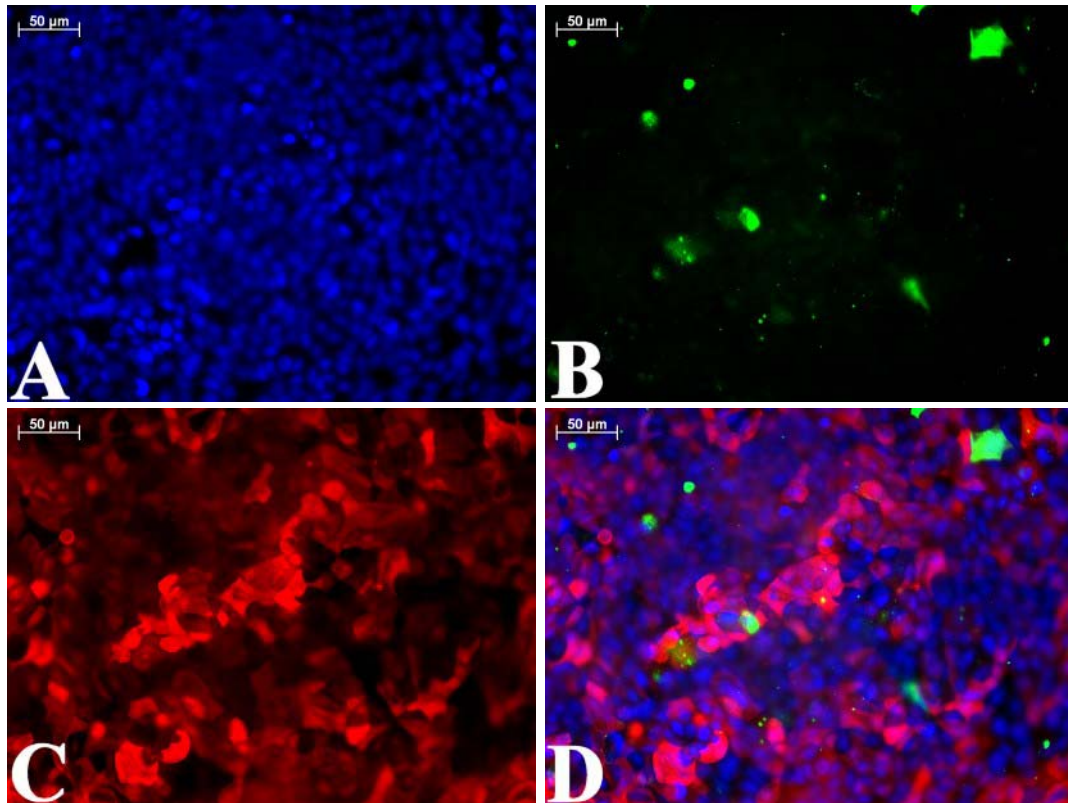


Figure 3.13. Sialyl Lewis A antigen expression on Caco-2 monolayers after coculture with 2×10^5 Raji B cells. A: Staining of cell nuclei with DAPI. B: Staining for sialyl Lewis A antigen with FITC. C: Staining of Caco-2 apical surface with rhodamine-UEA 1. D: Composite of figures A-C.

cells/filter led to significant breakdown of the monolayer. Caco-2 cells detached from large regions on each filter (Figure 3.14). Not surprisingly, monolayer electrical resistance also dropped sharply for filters with 2×10^6 Raji cells (Figure 3.15, triangles). Electrical resistance also dropped for filters incubated with 2×10^5 Raji cells (Figure 3.15, squares), suggesting that damage to the monolayer was occurring but was not yet pronounced enough to be revealed by microscopy (Figures 3.12 and 3.13).

3.4.3 Microsphere uptake experiments with cocultured monolayers

PLGA/PEMA microspheres with encapsulated FITC-BSA and surface-conjugated hIgA and HSA were suspended at 2 mg/ml in HBSS. An aliquot of the hIgA-conjugated microsphere suspension had free hIgA added to a concentration of 0.5 mg/ml to inhibit potential interactions between hIgA-conjugated microspheres and cell apical surfaces. Microsphere suspensions were transferred to wells of a 24-well plate, 600 μ l/well. Filters with Caco-2 monolayers cocultured for 2 days with 2×10^6 Raji B cells per filter were washed with HBSS and transferred to the wells containing microsphere suspensions. 150 μ l HBSS was added to the basolateral chamber of each filter. Filters were incubated at 37°C on an orbital shaker at 125 rpm. At selected time points up to 6 hours, 100 μ l HBSS from the basolateral chamber of each filter was collected and replaced with an equal volume of fresh HBSS. Basolateral fluid samples were then analyzed by flow cytometry to detect transcytosed microspheres. Each sample was run on the cytometer for a known length of time at the same flow rate, allowing for comparison of the different samples on a number of microspheres/unit volume basis.

The extent of monolayer damage after coculture with Raji cells was not known at the time the uptake experiment was performed. Clearly, with Caco-2 monolayers degraded as badly as Figure 3.14 indicates, transcytosis by M-like cells is impossible

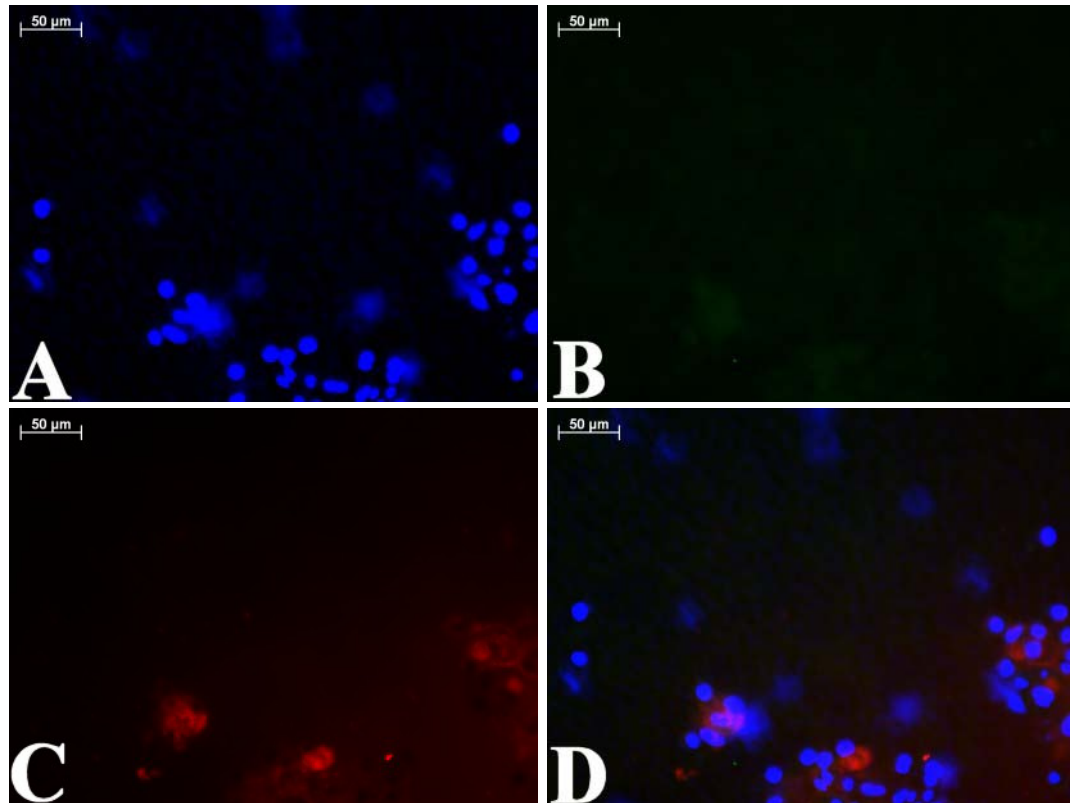


Figure 3.14. Degradation of Caco-2 monolayers after coculture with 2×10^6 Raji B cells. A: Staining of cell nuclei with DAPI. B: Staining for hIgA receptors with FITC. C: Staining of Caco-2 apical surface with rhodamine-UEA 1. D: Composite of figures A-C.

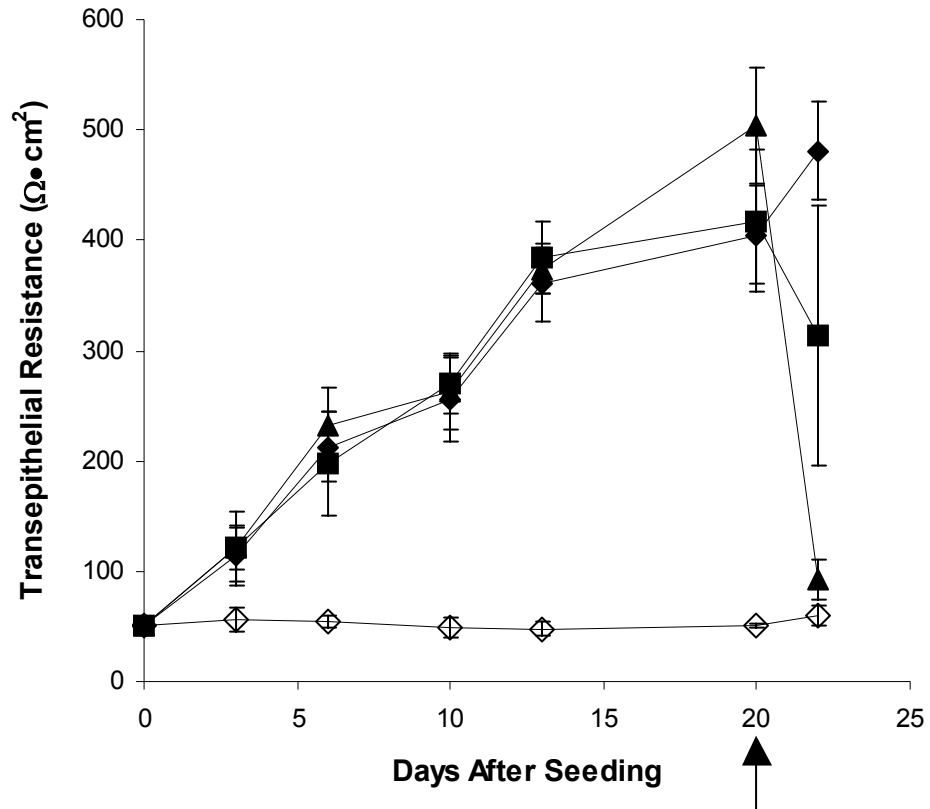


Figure 3.15. Effect of Raji B cells on electrical resistance of Caco-2 monolayers. Raji cells were seeded onto filters 20 days after Caco-2 cell seeding (arrow).
 ◆ = Monolayers with no Raji cells. ■ = Monolayers seeded with 2×10^5 Raji cells.
 ▲ = Monolayers seeded with 2×10^6 Raji cells. ◇ = Control filters with no cells.
 Data are means \pm standard deviations for individual measurements on filters at each time point ($n = 4$ filters for unseeded filters, $n = 6$ for each group of seeded filters).

to evaluate. Targeting of microspheres with specific surface ligands would not be measurable even if there was evidence that the monolayers expressed hIgA receptors, which is shown in Figure 3.12 not to be the case. Figure 3.16 shows the results of the microsphere transcytosis experiment. Microspheres incubated with cocultured monolayers were found in the basolateral fluid, most likely due to ordinary diffusion through the pores of the exposed filters. Monolayers not cocultured with Raji cells maintained high integrity, with very low levels of microsphere transcytosis, which is consistent with other microparticle uptake experiments [59,61]. Interestingly, the uptake curves obtained in this experiment are comparable with those of similar studies reported in the literature [59,61], despite the evident degradation of the monolayers.

3.5 Summary of in vitro microsphere targeting experiments

The results of experiments with PLGA/PEMA microspheres in the *in vitro* systems described in this chapter show that the ligands conjugated to the microspheres affect the interactions of the microspheres with the targeted surfaces. The model systems provide a controlled environment for evaluating these interactions, in which the observed increase in adhesion of the microspheres to targeted surfaces is clearly caused by specific ligand-receptor interactions. Ligand density on the microspheres is sufficient to enable targeting both in systems where contact between microspheres and receptors is virtually guaranteed, as in the case of the agarose columns, as well as in the open environment of the Caco-2 monolayers in which microspheres are freely suspended in solution.

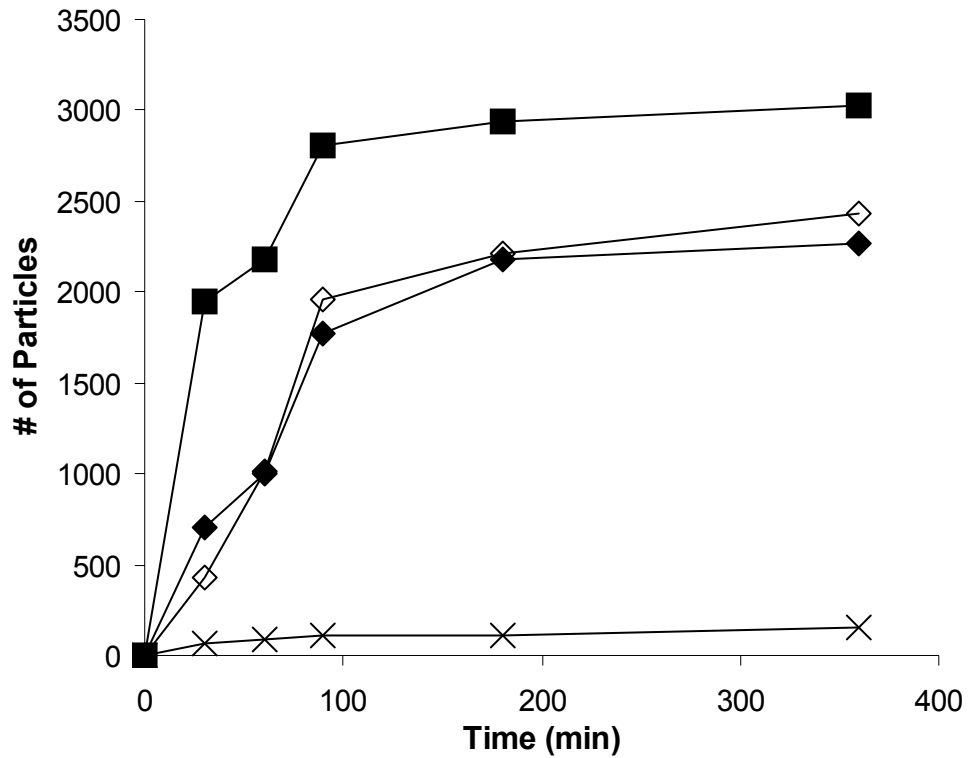


Figure 3.16. Transcytosis of PLGA/PEMA microspheres across Caco-2 monolayers cocultured with 2×10^6 Raji B cells. ■ = HSA-conjugated microspheres. ◆ = hIgA-conjugated microspheres. ◇ = hIgA-conjugated microspheres in 0.5 mg/ml hIgA. × = hIgA-conjugated microspheres on monolayers with no Raji cells.

CHAPTER 4

M CELL TARGETED MICROSPHERES IN MOUSE MODELS

4.1 Purpose

The *in vitro* model systems used in Chapter 3 to evaluate the effect of surface-conjugated ligands on microsphere behavior are simplifications of the environments these microspheres would encounter *in vivo*. To evaluate the effectiveness of surface ligands in targeting PLGA/PEMA microspheres to specific cell types *in vivo*, microspheres with surface-bound UEA 1 were orally administered to mice. UEA 1 has been shown to bind selectively to mouse M cells but not regular enterocytes [36,68], making it a suitable molecule for use in targeting microspheres to this cell type.

This chapter describes experiments using PLGA/PEMA microspheres with encapsulated protein to try to demonstrate targeting of these microspheres to M cells in mice. Microspheres with encapsulated FITC-BSA were used in uptake studies to attempt to detect microspheres in selected immunological tissues after oral administration. At selected time points after microsphere administration, mouse tissues were harvested and analyzed for the presence of microspheres. In order to demonstrate that targeting of microspheres to tissues not only increases their adhesion or uptake but also leads to improved clinical responses, microspheres with encapsulated OVA were used in an attempt to elicit anti-OVA antibody responses in serum and mucosal secretions in response to oral administration of microspheres. The goal of these experiments was to extend the results obtained in Chapter 3 to an *in vivo* environment, to show that conjugating an appropriate ligand to the surface of microspheres increases their uptake by the targeted M cells, and that the increase in uptake leads to improved immune responses.

4.2 Uptake of orally administered microspheres with encapsulated FITC-BSA in mouse tissues

Microspheres with encapsulated fluorescent label were orally administered to mice in an attempt to track microsphere uptake by M cells and transit to immunological tissues. Successful tracking of microsphere uptake would allow evaluation of the effect of microsphere-conjugated UEA 1 on microsphere uptake by M cells.

4.2.1 Microsphere administration and tissue collection

PLGA/PEMA microspheres with encapsulated FITC-BSA were suspended in 0.2 M sodium bicarbonate buffer at 50 mg/ml. Female BALB/c mice were fasted for 3 hr, and then 250 μ l of the microsphere suspension was administered by oral feeding needle. Mice were sacrificed by CO₂ inhalation at 1 and 4 days after microsphere administration. The spleen and mesenteric lymph nodes (MLN) were immediately harvested from each mouse. The small intestine was then washed with PBS to remove intestinal contents, and Peyer's patches were collected. Harvested tissues were disrupted on a metal sieve and passed through a 40 μ m cell strainer to create single-cell suspensions. Splenocytes were incubated in ACK lysis buffer (Quality Biological, Gaithersburg, MD) for 5 minutes at 4°C to lyse red cells. Cells were then centrifuged at 300x g for 5 minutes, and the supernatant was discarded. All cells were then suspended in 1% paraformaldehyde and analyzed by flow cytometry. Microspheres incubated in bicarbonate buffer for 1 and 4 days were used as controls for identifying microspheres during cytometry of the mouse tissue samples. Control tissues were harvested as described above from one mouse that did not receive a microsphere dose.

4.2.2 Uptake study results

Microspheres incubated in bicarbonate buffer for 1 and 4 days had essentially equal fluorescence, as shown in Figure 4.1. Data for 100,000 particles were collected on the cytometer for each tissue sample, which were then compared to the microsphere controls to determine the presence of microspheres in the samples. Figure 4.2 shows density plots of particle fluorescence vs. forward scatter for each of the tissue samples, as well as the microsphere controls. Microspheres had a minimum fluorescence intensity value of 100, which was used as a threshold value for determining the number of microspheres present in the tissue samples. Figure 4.2 shows that none of the tissue samples indicated the presence of microspheres. Repetition of this experiment with microspheres that had UEA 1 conjugated to the surface (prepared in section 3.3.1) had the same result.

4.3 Targeting of antigen-carrying microspheres to mouse M cells

To complement the uptake experiments described above, similar studies with antigen-encapsulated microspheres were performed. Microspheres were delivered orally, and both systemic and mucosal antibody responses to the encapsulated antigen were evaluated.

4.3.1 Production of PLGA/PEMA microspheres with encapsulated OVA for mouse M cell targeting

PLGA/PEMA microspheres with encapsulated OVA were generated by the method described in section 2.1, using 100 μ l of a 50 mg/ml OVA solution during the primary emulsion step. Microspheres had smooth, unbroken surfaces (Figure 4.3), and a mean diameter of 814 nm. Conjugation of UEA 1 and BSA to the microspheres was

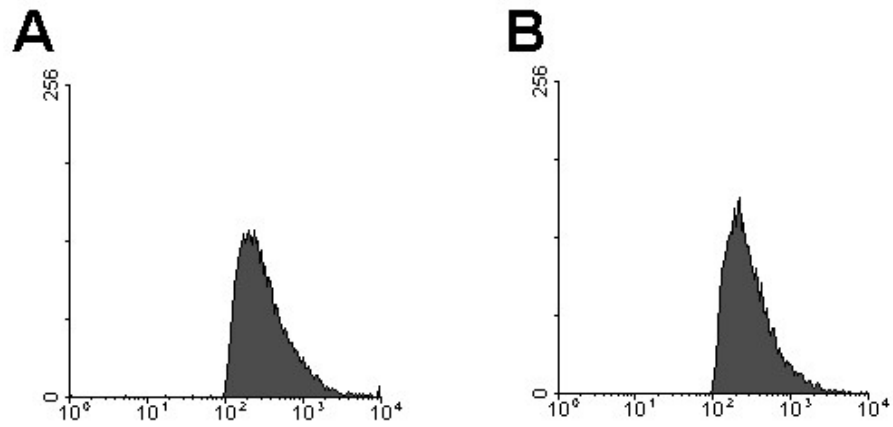


Figure 4.1. Fluorescence intensity of PLGA/PEMA microspheres with encapsulated FITC-BSA. A: Microspheres incubated 1 day in 0.2 M sodium bicarbonate. B: Microspheres incubated 4 days in 0.2 M sodium bicarbonate.

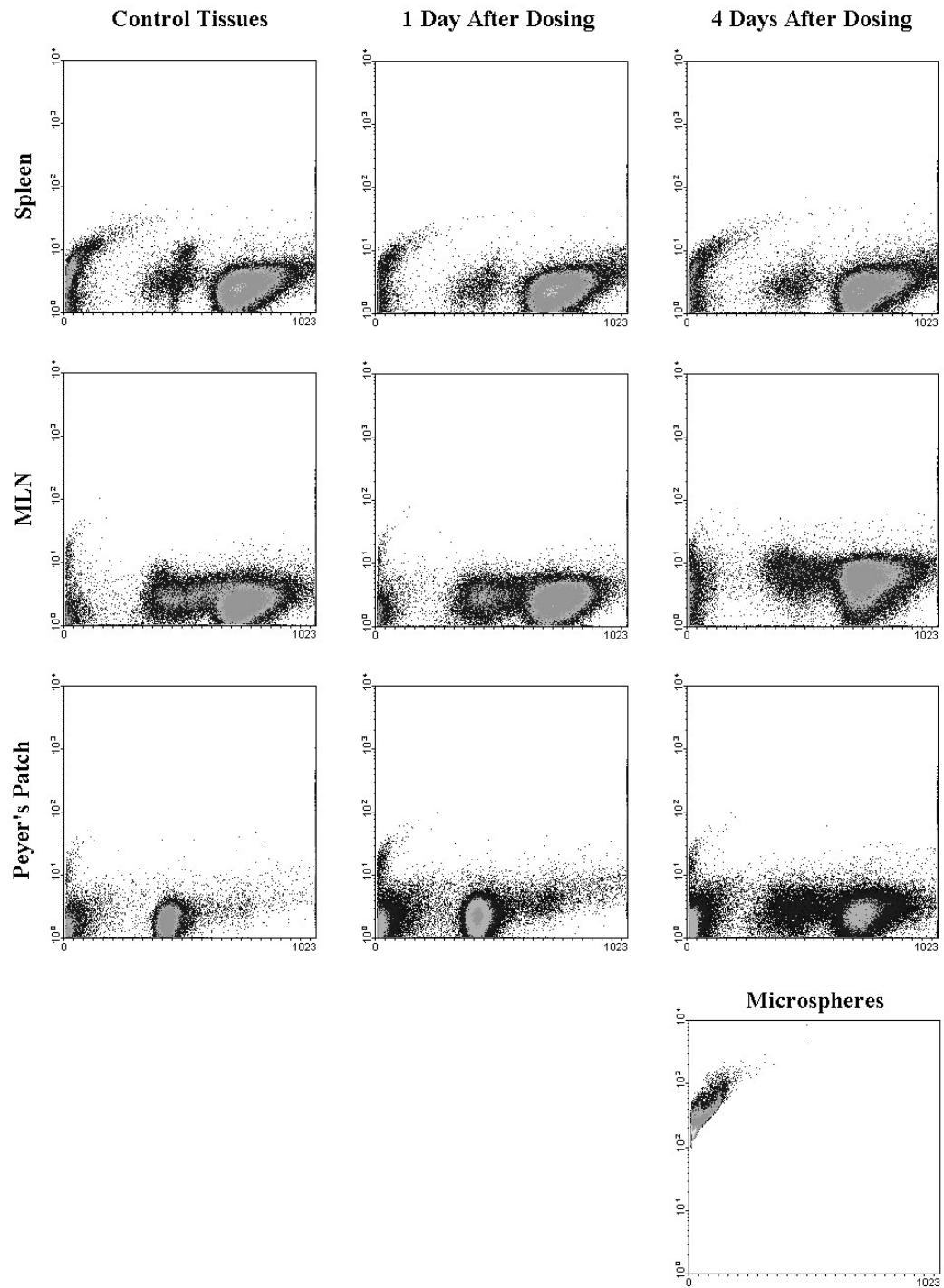


Figure 4.2. Mouse tissues analyzed for microsphere uptake by flow cytometry. Data are shown as density plots of fluorescence intensity vs. forward scatter.

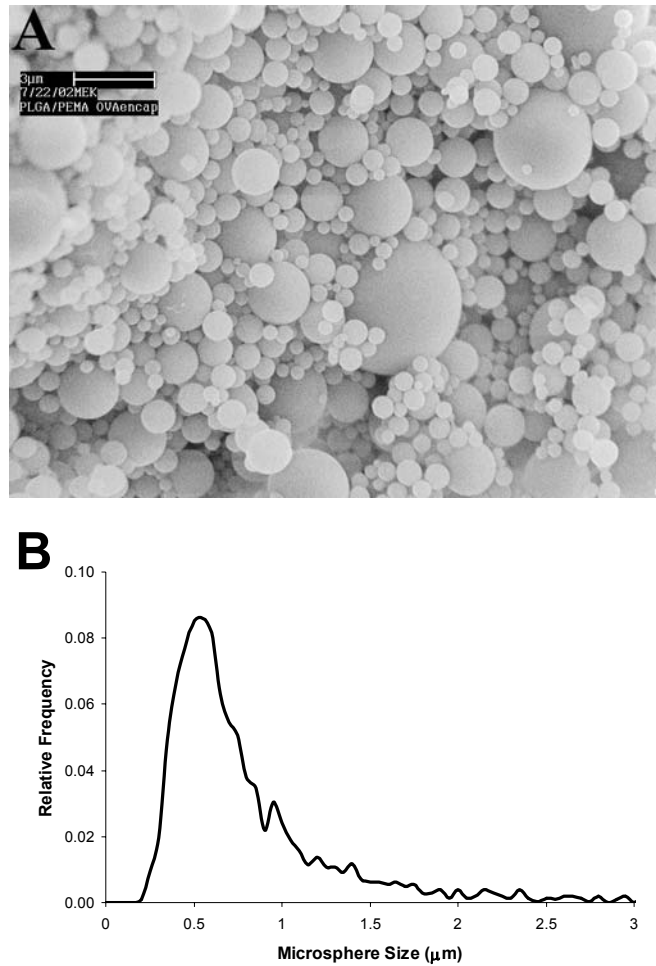


Figure 4.3. PLGA/PEMA microspheres with encapsulated OVA. A: Microsphere morphology. B: Microsphere size distribution (n = 2107).

performed according to the protocol described in section 2.3. During the ligand conjugation process, small aliquots of the microspheres were removed immediately before addition of UEA 1 or BSA. These aliquots were incubated overnight in borate buffer without ligand, but otherwise were processed identically to the microspheres conjugated to UEA 1 and BSA. These microspheres with no conjugated ligand were later used to determine the amount of encapsulated OVA lost during the conjugation process. Successful conjugation of the ligands to the microspheres was verified by immunocytometry (Figure 4.4), performed as described for microspheres with encapsulated FITC-BSA and conjugated BSA and UEA 1 in sections 3.2.2 and 3.3.1, respectively.

4.3.2 Determination of OVA loading level in microspheres

Total OVA loading of the microspheres before and after conjugation to UEA 1 and BSA was determined by bicinchoninic acid assay (microBCA™, Pierce, Rockford, IL). Microspheres were dissolved in 0.5 M NaOH at a concentration of 5 mg/ml. After microspheres were dissolved, the samples were neutralized with an equal volume of 0.45 M HCl in PBS. The amount of acid added to neutralize the solutions was slightly less than the stoichiometric amount because the microspheres produce lactic and glycolic acid during degradation. In addition, the assay is more compatible with slightly basic samples than slightly acidic ones. A diluent solution for OVA standards was prepared in such a way as to match the processing of the microsphere samples as much as possible. PLGA/PEMA microspheres with no encapsulated or conjugated proteins were dissolved in 0.5 M NaOH at a concentration of 5 mg/ml and then neutralized with an equal volume of 0.45 M HCl in PBS. This neutralized solution of dissolved microspheres was then used to prepare OVA standards based on a 250 µg OVA/ml starting solution, serially diluted 1:2 down to

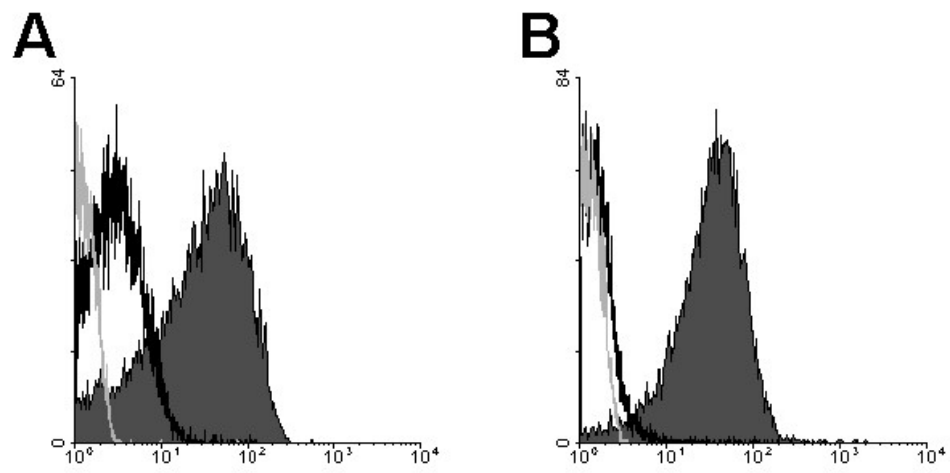


Figure 4.4. Detection of (A) UEA 1 and (B) BSA conjugated to the surface of PLGA/PEMA microspheres with encapsulated OVA. Gray line: microspheres before incubation with antibodies. Black line: microspheres incubated with irrelevant primary antibody (control for non-specific antibody adsorption). Solid fill: microspheres incubated with primary antibody specific for conjugated protein.

0.98 μg OVA/ml. Samples and standards were applied in triplicate to 96-well plates, 150 μl /well. Assay working reagent was prepared according to manufacturer's instructions, and 150 μl was added to each well. Plates were covered and incubated at 37°C for 2 hours. After incubation the cover was removed, and optical density of each well at 562 nm was measured on a ThermoMax 96-well plate reader (Molecular Devices, Sunnyvale, CA). Averages were calculated for the triplicate wells of each sample and standard.

A plot of OVA concentration vs. optical density for the standards produced a linear standard curve. Comparison of optical density values of microsphere samples to the standard curve indicated that the microspheres contained 13.7 μg OVA/mg microspheres before conjugation to UEA 1 and BSA. Microspheres undergoing the conjugation protocol but not incubated with UEA 1 or BSA had their OVA loading reduced to 5.3 μg /mg microspheres, a loss of 61% of the original encapsulated protein. Microspheres with UEA 1 and BSA conjugated to the surface showed no detectable increase in total protein compared to the microspheres not incubated with protein during the ligand conjugation process.

4.3.3 Immunization of mice with microspheres containing OVA

PLGA/PEMA microspheres with encapsulated OVA and either UEA 1 or BSA conjugated to the surface as described in section 4.3.1 were suspended in 0.2 M sodium bicarbonate buffer to produce suspensions that contained a total of 400 μg /ml OVA encapsulated in microspheres. Another suspension of UEA 1-conjugated microspheres was prepared in a solution of 500 mM L-fucose, 0.2 M sodium bicarbonate buffer. Microsphere suspensions were administered by oral feeding needle, 500 μl /mouse, for a dose of 200 μg OVA in microspheres/mouse, with groups of 5 mice receiving each of the 3 microsphere suspensions. Mice were female

BALB/c, 10 weeks old at the time of feeding. As a control to verify the immunogenicity of the OVA encapsulated inside the microspheres, another group of 5 mice received microspheres by subcutaneous injection. These microspheres had UEA 1 conjugated to the surface, and were suspended in PBS to produce suspensions containing 200 µg/ml OVA in microspheres. Mice receiving injections were each given 250 µl of this suspension, for an OVA dose of 50 µg encapsulated inside microspheres. A final group of 5 mice received no immunization (naïve controls). Ten weeks after the initial dose, microsphere administration was repeated for the 3 groups receiving microspheres orally. The groups and administered microspheres are listed in Table 4.1.

Table 4.1. Summary of Immunizations with Targeted PLGA/PEMA Microspheres with Encapsulated OVA

Group #	Administration Route	Microsphere Ligand	500 mM L-fucose	OVA Dose (µg)	Booster at Week 10 (µg OVA)
1	Oral	UEA 1	+	200	200
2	Oral	UEA 1	-	200	200
3	Oral	BSA	-	200	200
4	Subcutaneous	UEA 1	-	50	N/A
5	None	N/A	-	N/A	N/A

4.3.4 Collection and processing of samples for ELISA

Mice were transferred to fresh individual cages, where they remained until they had dropped a sufficient quantity of feces (~100 mg), which was immediately collected into microcentrifuge tubes. Mice were then anesthetized with isoflurane in an induction chamber, and anesthesia was maintained at 2.5% isoflurane in oxygen, administered by precision vaporizer. Vaginal lavage was performed on each

anesthetized mouse by introduction of 20 μ l of PBS into the vagina with a blunt micropipette tip. PBS was pipetted in and out of the vagina 20 times. Lavage was repeated with 20 μ l of fresh PBS, for a total of 40 μ l lavage fluid per mouse. Blood was then collected from the retro-orbital sinus by penetration with 50 μ l glass capillary tubes. One hundred microliters of blood was collected per mouse. Mice were then removed from the vaporizer, and bleeding was stopped by application of gentle pressure with a gauze pad. Mice were returned to cages when ambulatory. Pre-immune samples were collected before immunization, and then after immunization at regular intervals.

Blood and lavage fluids were stored overnight at 4°C. These samples were then centrifuged at 10,000x g for 10 minutes at 4°C. Serum and lavage supernatants were then collected from each sample and stored at -80°C until analyzed for antibodies by ELISA. Feces samples were suspended at 10% w/v in protease inhibitor cocktail (Roche, Indianapolis, IN) in PBS, supplemented with 10 μ g/ml pepstatin A (Sigma). Soluble protein was extracted from the fecal pellets by heavy vortexing of the samples to break up the pellets. Samples were then centrifuged at 10,000x g for 5 minutes, and extracts were collected from the supernatant and stored at -80°C until analysis by ELISA.

4.3.5 Measurement of anti-OVA antibodies by ELISA

Each well of an Immulon 4HBX 96-well plate (Dynex, Chantilly, VA) was coated with 100 μ l of a 5 μ g/ml solution of either OVA or UEA 1 in 0.05 M carbonate buffer, pH = 9.6, covered, and incubated overnight at 4°C. The plate was then washed once with 0.05% Tween-20 in PBS (wash solution). Then, the plate was blocked with 300 μ l/well of either 3% BSA in PBS (OVA-coated plates) or 3% BSA, 200 mM L-fucose in PBS (UEA 1-coated plates) and incubated for 4 hours at room temperature.

The L-fucose was added to inhibit binding of the lectin UEA 1 to carbohydrate residues on antibody molecules, which causes unacceptably high background readings for the plate. After blocking, the plate was washed 4 times with wash solution. Samples (serum, lavage fluid, feces extract) were diluted in 0.05% BSA in PBS (for anti-OVA antibody detection) or 0.05% BSA, 200 mM L-fucose in PBS (for anti-UEA 1 antibody detection) and applied in duplicate to wells, 100 μ l/well. Dilutions of samples were 1:5 for feces extracts, 1:50 for lavage fluid, and 1:100 for serum. Plates were incubated overnight at 4°C. After incubation, plates were washed 4 times with wash solution. Alkaline phosphatase-conjugated goat-anti-mouse IgG and goat-anti mouse IgA (Zymed) were diluted 1:200 in 0.05% BSA in PBS (for OVA plates) or 0.05% BSA, 200 mM L-fucose in PBS (for UEA 1 plates). One hundred microliters of the appropriate enzyme-conjugated antibody solution was added to each well, and incubated for 1 hour at room temperature. Plates were then washed 4 times with wash solution. One hundred microliters of 1 mg/ml p-nitrophenyl phosphate substrate in 0.2 M Tris buffer (Sigma) was added to each well. Plates were incubated for 30 minutes at 37°C to allow for substrate conversion, and the reaction was then quenched by addition of 100 μ l/well of 1 M NaOH. Optical density of each well at 405 nm was then measured, and the average of the duplicate wells for each sample was calculated.

No antibody responses were detected in response to the initial dose of orally administered microspheres, as shown in Figure 4.5. This figure shows that 7 weeks after immunization the ELISA results for samples from mice receiving microspheres orally are equivalent to those of unimmunized controls. For mice receiving microspheres subcutaneously, serum IgG anti-OVA and anti-UEA 1 responses were observed, with 3 of the 5 mice producing serum samples giving OD values over 2.0 for both OVA and UEA 1 antibodies. The oral booster dose given at week 10 had no noticeable effect, as the ELISA results for samples collected at week 12 show no

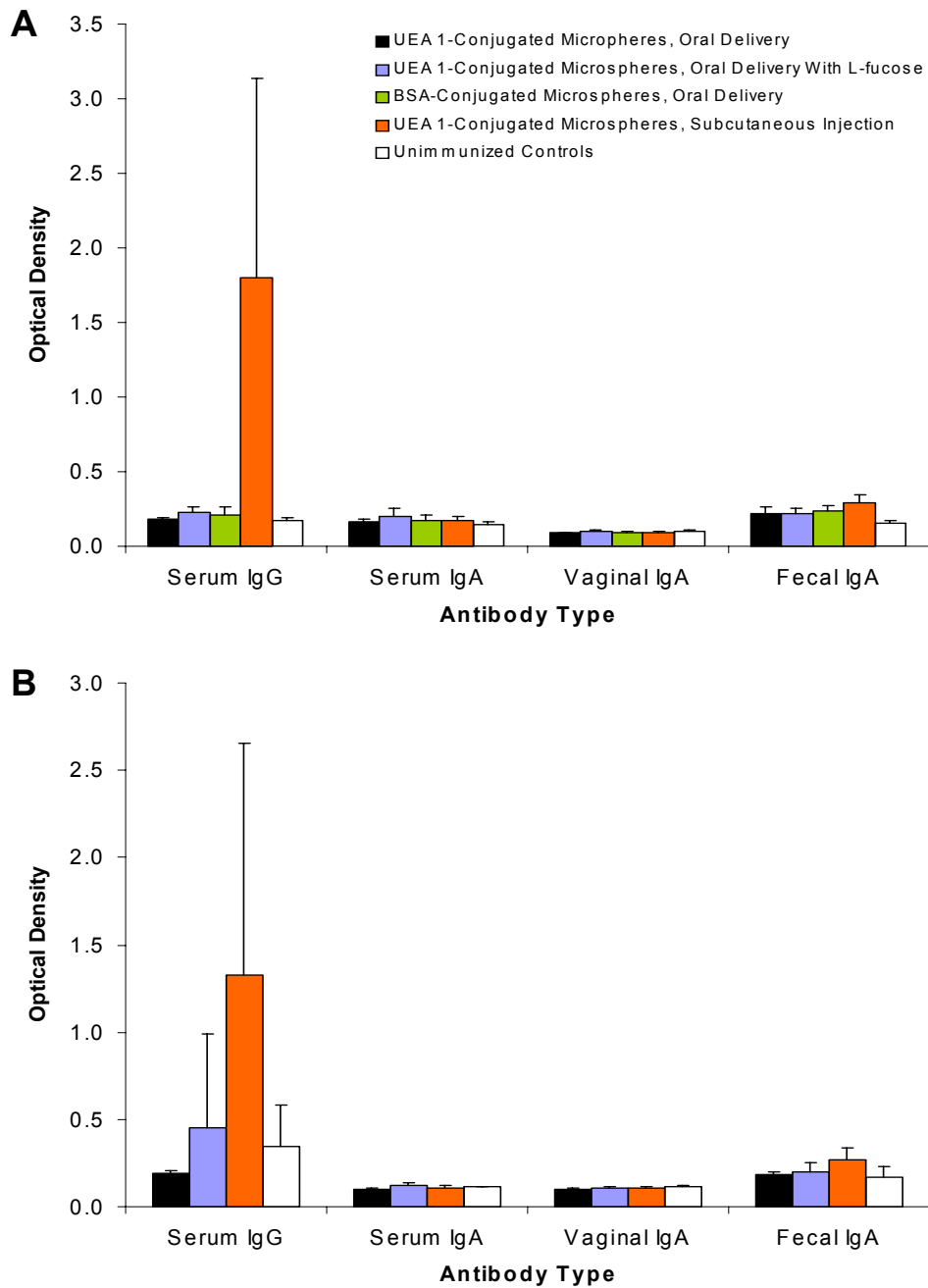


Figure 4.5. ELISA results for mouse samples collected 7 weeks after immunization. A: Anti-OVA response. B: Anti-UEA 1 response. Data are means \pm standard deviation for 5 mice in each experimental group.

increase in OD for orally immunized mice compared to naïve controls (Figure 4.6). At week 12, 4 of the 5 subcutaneously immunized mice generated strong serum IgG anti-OVA responses, and 3 of 5 had strong serum IgG anti-UEA 1 responses.

4.3.6 High-dose immunization of mice with OVA encapsulated in PLGA/PEMA microspheres

With the immunization study with targeted microspheres showing no detectable antibody responses in any of the groups receiving microspheres orally, an attempt was made to determine the minimum dose of OVA-encapsulated microspheres required to produce detectable antibody responses. Mice from the unimmunized control group of the initial immunization study described in section 4.3.3 were orally immunized with PLGA/PEMA microspheres containing OVA, but not conjugated to any surface ligands. Individual mice received from 400 to 1200 µg of OVA in microspheres. Mice received oral booster doses of the same microspheres 6 weeks after the initial dose, in an amount equal to one-half the initial dose (200 to 600 µg OVA).

Figure 4.7 shows the results of ELISA assays on samples collected 5 and 10 weeks after the initial immunization. Serum samples showed no significant increase in OD compared to an unimmunized control. The anti-OVA fecal IgA response is modest at best, with OD readings at week 12 that are slightly higher than the unimmunized control. The intensity of this response does not correlate with the size of the administered OVA dose, however, making conclusions difficult to draw. Anti-OVA IgA in the vaginal lavage fluid was not detectable at week 5. Lavage fluid could not be collected at week 12 due to an outbreak of mouse parvovirus (MPV) in the animal housing facility. The effect this outbreak may have had on the study is unclear, but MPV is known to perturb immune system function [69]. No matter the effect of

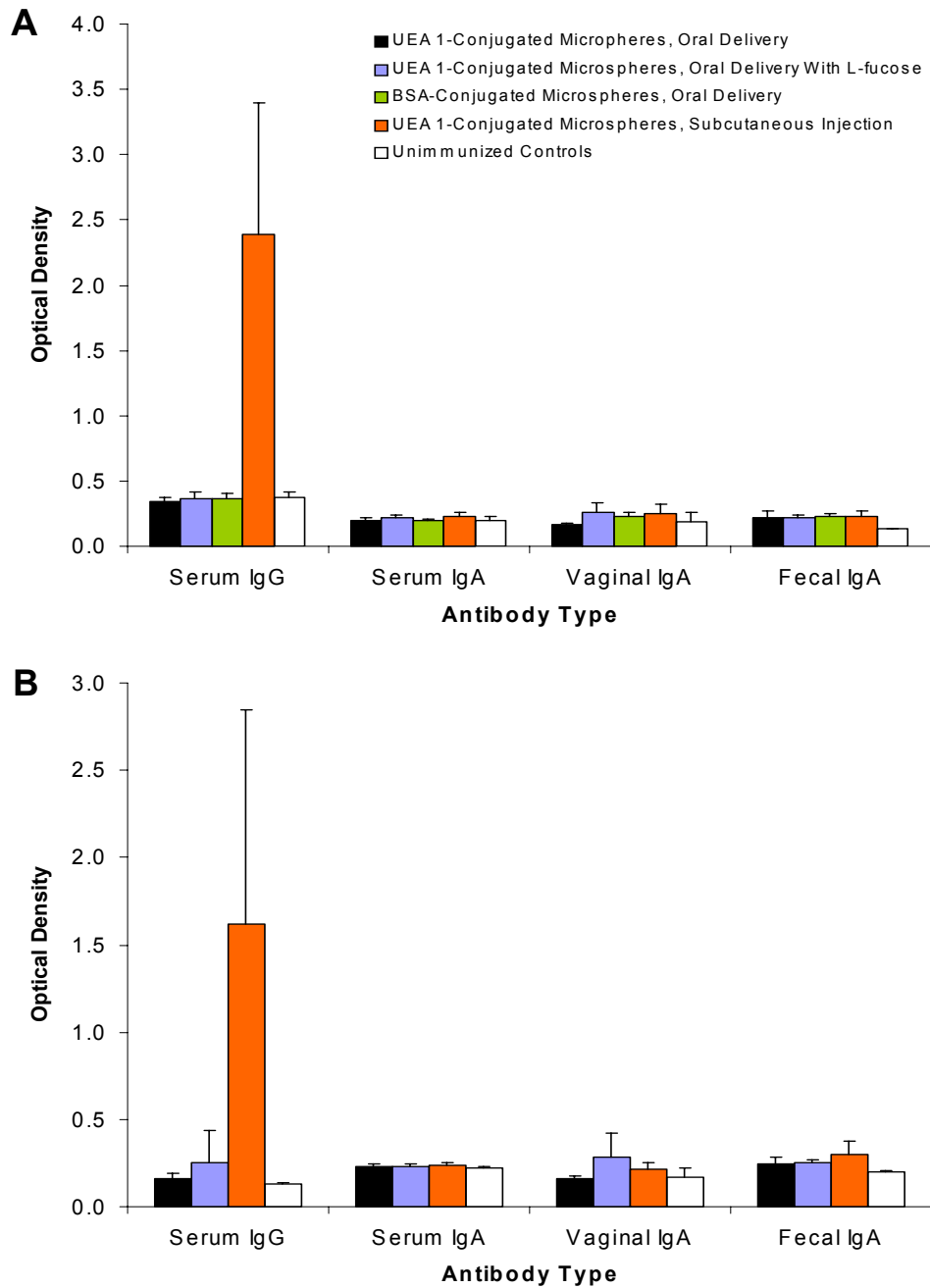


Figure 4.6. ELISA results for mouse samples collected 12 weeks after immunization. A: Anti-OVA response. B: Anti-UEA 1 response. Data are means \pm standard deviation for 5 mice in each experimental group.

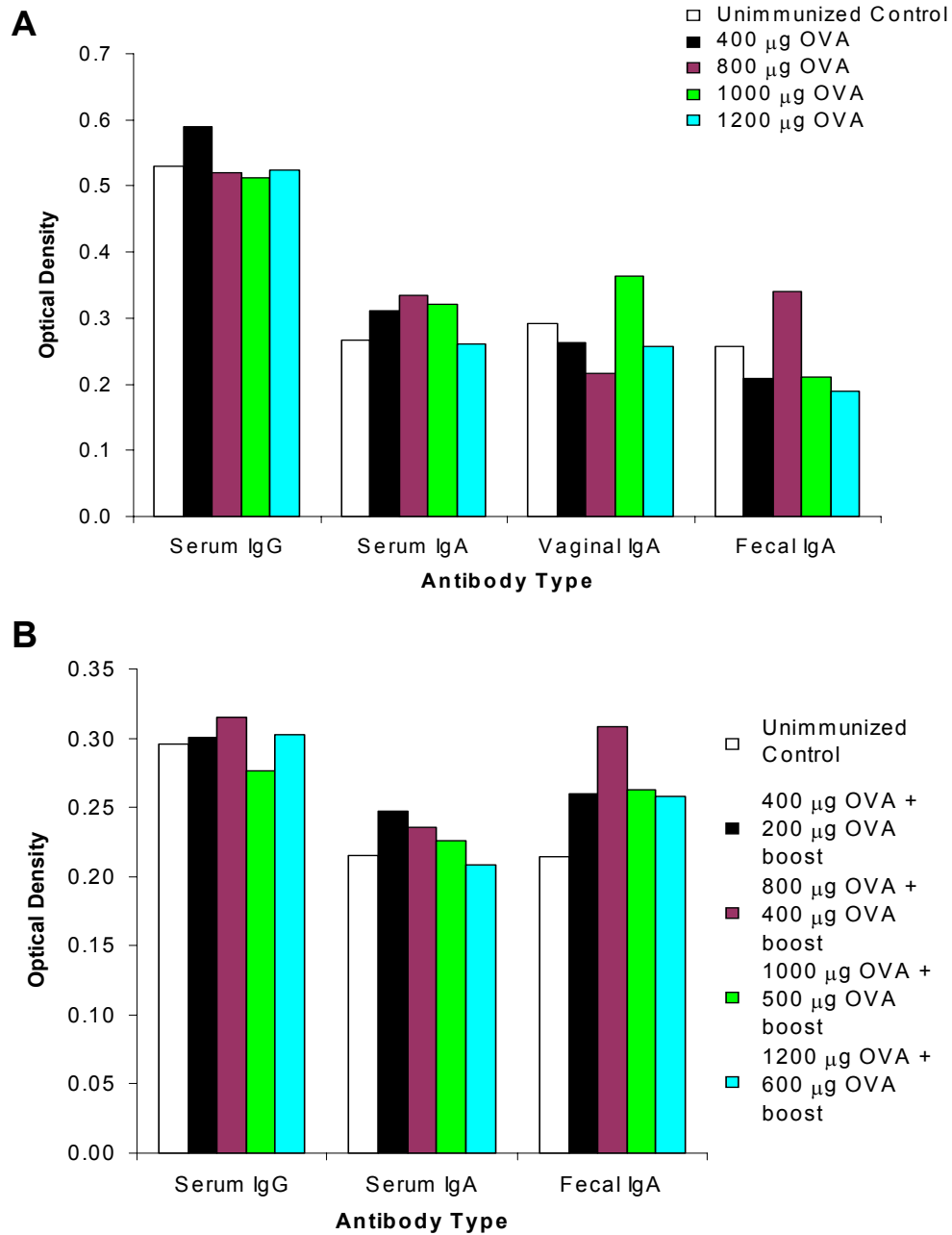


Figure 4.7. Anti-OVA response to high doses of OVA encapsulated in PLGA/PEMA microspheres. A: 5 weeks after immunization. B: 10 weeks after immunization.

the viral outbreak, a minimum required dose of OVA in PLGA/PEMA microspheres should be determined with untargeted formulations before attempting to repeat the immunization experiment with UEA 1-conjugated microspheres.

4.4 Conclusions for mouse M cell targeting studies

Neither the microsphere uptake or immunization studies described in this chapter produced interpretable results. Flow cytometry did not reveal the presence of microspheres in the spleen, MLN, or Peyer's patches of mice at 1 or 4 days after oral administration. Mice orally immunized with up to a total of 1.8 mg OVA (initial + booster doses) in microspheres showed no serum or mucosal antibody responses. Targeting of smaller OVA doses in UEA 1-conjugated microspheres also failed to generate measurable antibody responses.

Using flow cytometry to detect microspheres in tissues after oral administration has been described previously [70]. In that instance, polystyrene microspheres were used, enabling tissues to be dissolved in a solution of 1% Triton X-100 and 1% KOH. Such conditions are not appropriate for PLGA microspheres, as the alkaline environment would lead to rapid microsphere degradation. The incubation in Triton X-100/KOH is in effect a purification step, as it selectively eliminates cells from the samples and therefore increases the (low) probability that events detected by the flow cytometer will be microspheres. For tissue samples with very low concentrations of microspheres such as the ones analyzed in uptake experiments, this purification may have made the difference between detecting a few rare fluorescent microspheres in the sample and not detecting any. With the restrictions on sample handling to prevent PLGA microsphere degradation, it might be advisable to seek alternate, simpler methods to detect microsphere adhesion and uptake by M cells. A possible technique would be to harvest sections of small

intestine containing Peyer's patches for fluorescent microscopy at selected time points after oral administration of microspheres. This histological method would provide less quantitative data compared to flow cytometry, but would allow for direct visualization of the cells being targeted.

A number of reports describe the generation of systemic and mucosal antibody responses to OVA in orally administered PLGA microspheres [25-27]. These studies all use microspheres with no surface ligands. Total OVA doses ranged from 600 μg [25] to 6 mg (3 mg initial dose, 3 mg booster at 4 weeks) [27]. Sample collection and ELISA methods were similar to those described in sections 4.3.4 and 4.3.5. The observed responses were not strong, especially considering the amount of antigen delivered, but they were clearly detectable. The robust serum IgG response to OVA in the group receiving microspheres by subcutaneous injection (Figures 4.5 and 4.6) shows that the OVA remains immunogenic through the encapsulation and ligand conjugation protocols. If encapsulated OVA doses larger than those used in section 4.3.6 are required in order to generate observable antibody responses, preparing sufficient amounts of UEA 1-conjugated microspheres for evaluating the ability of M cell targeting to increase the efficiency of oral antigen delivery will be a costly endeavor.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

5.1 Conclusions

Replacing PVA with PEMA during PLGA microsphere production resulted in microspheres with a substantially enhanced capacity for surface ligand conjugation by carbodiimide cross-linking. Switching between the two surfactants had no significant effect on microsphere size or morphology. Perhaps more importantly, the release kinetics of encapsulated protein are unchanged for PLGA/PEMA vs. PLGA/PVA microspheres.

The carboxylic acid groups are present on the surface of PLGA/PEMA microspheres at a similar density to that of the carboxylated polystyrene microspheres that are currently used as model particles for microsphere targeting experiments, as demonstrated by the comparable fluorescence intensities of the two microsphere types after conjugation to 5-(aminoacetamido)fluorescein (Figure 2.7). Binding of this fluorescent ligand to the microspheres was due to covalent conjugation, not surface adsorption, as shown in Figure 2.6. Microspheres of PLGA/PVA and PLGA-COOH/PVA showed only limited capacities for ligand conjugation. For PLGA/PVA microspheres this result was expected, as there are no carboxylic acid groups available for ligand conjugation with this microsphere formulation. The PLGA-COOH/PVA microspheres, however, have carboxylic acid groups at the end of PLGA polymer chains. The low levels of ligand conjugation to these microspheres compared to PLGA/PEMA could be caused by blocking of available carboxylic acid groups by PVA at the microsphere surface. Another possible explanation is that because carboxylic acid groups are present along the entire length of each PEMA

molecule instead of only at the ends, there are simply more total carboxylic acid groups present in PLGA/PEMA microspheres as compared to PLGA-COOH/PVA.

The amide bonds formed between microspheres and conjugated ligands show excellent stability in aqueous solution, with approximately 90% of the conjugated ligand still present on the microsphere surface after over a month of incubation in buffered solution. The stability of the microsphere-ligand linkage will enable successfully targeted microspheres to remain at the desired location for the full duration of payload release. For applications in which the microsphere payload has undesirable side effects, such as targeting of chemotherapy drugs to tumors, this stability is especially desirable, as it will help to hold the microspheres at the desired location. Interestingly, comparison of surface ligand (Figure 2.10) and encapsulated protein release (Figure 2.9) curves indicates that conjugated ligand remains at the microsphere surface even after release of encapsulated protein has ceased.

The microspheres released a significant amount of encapsulated protein during ligand conjugation. Microspheres with encapsulated OVA that were conjugated to BSA and UEA 1 in section 4.3.1 lost 61% of their encapsulated protein during the conjugation process. Microspheres with encapsulated FITC-BSA lost a similar fraction of their encapsulated protein during ligand conjugation (Figure 2.9, open symbols). The conjugation protocol exposed the microspheres to aqueous buffer for approximately 20 hours, during which time the microspheres released encapsulated protein. The conjugation process did not change the kinetics of payload release for the microspheres, as the difference in release profiles in Figure 2.9 for PLGA/PEMA microspheres with conjugated ligands versus those not exposed to the conjugation protocol is simply a shift of the release curve forward by 20 hours.

In addition to the model ligand 5-(aminoacetamido)fluorescein, which is a small molecule (MW = 404.4), macromolecules can also be conjugated to

PLGA/PEMA microspheres, as evidenced by the variety of proteins coupled to the microspheres in Chapters 2 and 3. The detection of these conjugated proteins with labeled antibodies shows that the three-dimensional structure of the ligands was maintained through the conjugation process, at least at the epitopes recognized by the antibodies. As the binding activity of the ligands is dependent on their three-dimensional structure, it is critical that the conjugation process changes ligand structure as little as possible.

The *in vitro* systems used to evaluate microsphere targeting to receptor-bearing surfaces in Chapter 3 further demonstrated that the conjugated ligands retained their binding activity, and also were present at sufficient density to affect the way the microspheres interacted with the targeted surfaces. When the microspheres were loaded onto agarose columns, a significant fraction of the microspheres was retained in the column, regardless of the particular combination of microsphere-coupled ligand and agarose-bound receptor. This is not surprising, as the agarose beads form a tight matrix that could be expected to physically impede the flow of microspheres no matter the particular ligand/receptor pair being evaluated in a given trial. The addition of denaturing 6 M guanidine HCl to the columns did not result in any additional elution of BSA-conjugated microspheres from the agarose-biotin column or streptavidin-conjugated microspheres from the agarose-mouse IgG column. This observation supports the explanation that much of the microsphere load placed on each column becomes physically trapped within the column, for any ligand/receptor combination being investigated.

By matching the ligand on the microspheres with the proper receptor on the agarose matrix, however, microsphere retention was increased, as demonstrated by the significant increase in the fraction of streptavidin-conjugated microspheres retained on an agarose-biotin column (Figure 3.2, open squares). A similar increase in

microsphere retention was seen on agarose-mouse IgG columns using microspheres conjugated to goat-anti-mouse IgG (Figure 3.3). Antibodies have previously been generated for the purpose of targeting microspheres to specific cell types [54], suggesting that this is a reasonable model ligand/receptor binding pair which could later be substituted with microsphere-bound antibodies specific for clinically relevant receptor molecules.

Compared to the agarose columns, the experiments with Caco-2 cell monolayers provide a very different environment for evaluating the ability to target PLGA/PEMA microspheres to a selected surface. Caco-2 monolayers have been incubated with ligand-bearing microspheres in other studies [17,52], with the result being increased association of the targeted microspheres with the monolayers as compared to untargeted microspheres. In these studies, the monolayers were at the bottom of the incubation chambers, so that microspheres settling to the bottom of the chamber during incubation had extended direct contact with the monolayer surface. In the system described in Chapter 3, the cell monolayer is inverted and suspended near the top of the incubation medium, so that the monolayer only encounters microspheres freely suspended in solution. The results show that conjugating the ligand UEA 1 to the microsphere surface doubles the adhesion density of the microspheres to the apical surface of the Caco-2 cells (Figure 3.8). This increase was due to specific interaction of the microsphere-bound lectin with receptors on the apical surface of the monolayer, as incubation of the lectin-bearing microspheres with the cells in the presence of the inhibitory sugar L-fucose reduced adhesion density to the same level as for BSA-conjugated control microspheres. This model system is a simplification of the intestinal environment *in vivo*, most notably in the absence of the continually renewing mucus layer present along the gastrointestinal tract, but it does demonstrate

that microspheres freely suspended in solution can be targeted to epithelial cell surfaces with appropriate ligands.

5.2 Recommendations for future work

The characterization of PLGA/PEMA microspheres presented in Chapter 2 compares microspheres of PLGA/PEMA to those of PLGA/PVA. In addition, the comparison of fluorescence intensity of PLGA/PEMA and carboxylated polystyrene microspheres indirectly indicates that they have similar densities of available surface carboxylic acid groups. Direct methods for characterizing the density of surface functional groups, such as zeta potential measurements or X-ray photoelectron spectroscopy [14,50] would be of value. Making changes in the PLGA/PEMA microsphere production process might result in a further increase in surface carboxylic acid group density, and direct analysis by the methods mentioned would enable this to be evaluated.

These methods could also be used to investigate the effect of surfactants other than PEMA on microsphere properties. That switching from PVA to PEMA resulted in such minor changes to the resulting microspheres (apart from the desired change in functional groups at the microsphere surface) suggests that other surfactants could be successfully used as well. Other polymers with backbones of carbon-carbon bonds and hydrophilic side chains should effectively stabilize the emulsion during microsphere production, and by selection of a molecule with the appropriate side chains a variety of different hydrophilic functional groups could be incorporated into the microsphere surface. In situations where cross-linking chemistries other than carbodiimide coupling are desired, microspheres made with the appropriate surfactant would provide the necessary functional groups for the selected conjugation method.

It is important to quantify the extent of ligand binding to the microspheres. The level of 5-(aminoacetamido)fluorescein conjugated to the PLGA/PEMA microspheres could be estimated by comparison to standard beads as analyzed by flow cytometry. Detection of protein ligands by flow cytometry, however, while it provides clear evidence of the presence of the ligands on the microsphere surface, is not quantitative. Many of the antibodies used for detection of the conjugated proteins are polyclonal, including the PE-labeled secondary antibodies. Uncertainty about the nature of the interaction between these polyclonal antibodies and their antigens (such as the number of epitopes on each antigen molecule recognized by the polyclonal mixture) makes determination of the number of conjugated ligands from cytometer fluorescence intensities difficult. Efforts to quantify the amount of protein conjugated to the microspheres with the microBCA™ total protein assay were not successful, because the amount of conjugated protein was below the detection limit of the assay. Other colorimetric protein assays have similar detection limits, so alternative methods are needed. Flow cytometry could be used [71,72], with monoclonal primary and secondary antibodies in order to obtain reliable estimates of conjugated ligand levels.

Identifying a technique that will allow quantification of the extent of protein ligand conjugation to the PLGA/PEMA microspheres would be helpful in optimizing the conjugation protocol. The protocol used for conjugating ligands to the microspheres was designed for carboxylated polystyrene microspheres. Since it was not originally intended for degradable microspheres, or for microspheres carrying encapsulated drugs, the duration of the various incubation steps was set without regard for premature microsphere degradation or payload release. The results described in Chapter 2 show that substantial amounts of encapsulated protein are released during the ligand conjugation process. While the microspheres still retain significant amounts of encapsulated material, this loss of payload is wasteful, and could likely be

moderated with simple changes to the conjugation protocol. Incubation lengths are one process parameter worth investigating, as is the relative concentration of EDC, microspheres, and ligands. Since microsphere degradation is accelerated in both acidic and alkaline environments, adjustment of the pH of the buffer solutions used during ligand conjugation may also affect payload release during ligand conjugation. Many of the agents considered for delivery via microspheres have a very high monetary value, so efforts that lead to reduction in payload loss during ligand conjugation would be worthwhile.

Some changes to the conjugation protocol that reduce premature payload release (such as shortening incubation times) are likely to also reduce the extent of ligand conjugation. A balance will need to be found between ligand conjugation and microsphere payload retention. The necessary ligand surface density to achieve successful microsphere targeting will likely vary depending on the application. *In vitro* models are able to show that PLGA/PEMA microspheres have sufficient surface ligand capacity to target them to surfaces, at least in carefully controlled systems. Conditions *in vivo* are far more complex, and cannot truly be duplicated *in vitro*. The *in vivo* experiments summarized in Chapter 4 are inconclusive with regards to microsphere targeting to M cells. As discussed in section 4.4, it may be valuable to begin with simpler *in vivo* microsphere uptake models than those used in Chapter 4. Before attempting to show increased uptake of targeted microspheres in distant tissues such as the spleen or MLN, visualizing Peyer's patches by microscopy after microsphere administration would show if targeted microspheres are at least adhering to M cells at higher levels than untargeted ones.

PLGA microspheres with surface-conjugated ligands may be useful for applications other than targeted drug delivery. Specifically, these microspheres could be used as scaffolds for tissue engineering. Influencing cell attachment and growth on

microspheres with surface ligands has been pursued with systems such as PLGA/PVA microspheres with adsorbed poly(lysine) [73] and poly(ethylene glycol-di-methacrylate-co-hydroxyethyl methacrylate) microspheres conjugated to fibronectin and collagen by glutaraldehyde [74]. PLGA/PEMA microspheres could be used in similar ways, serving as a biodegradable scaffold to which ligands promoting cell attachment could be covalently conjugated. Porous microspheres can be produced by addition of salts such as NaCl or CaCl₂ to the inner aqueous phase during emulsification [75]. If PEMA is also added to the inner aqueous phase, these pores would then be lined with carboxylic acid groups through the same surfactant partitioning that leads to carboxylic acid groups at the microsphere surface when PEMA is used as the stabilizer during the second emulsification step. Peptide sequences known to promote cell adhesion and aggregation such as RGD or YIGSR [76] could then be conjugated to microspheres, via the peptide N-terminus. If linkage to the N-terminus is not suitable, the peptides could be produced with lysine residues at one end to provide alternate amines for carbodiimide conjugation.

In addition to the conjugated cell attachment promoters, the microspheres could be produced with encapsulated growth factors, which would release over time and further promote cell growth onto and into the porous microspheres. For this application, the ideal microsphere size is likely to be much larger than the ~ 1 μm spheres discussed in this dissertation. Reducing the duration of the sonication steps or changing other process parameters would result in microspheres on the order of tens to hundreds of micrometers in diameter, which would be more appropriate for use as cell scaffolds. By using PEMA that has already been conjugated to the desired peptides as the surfactant during microsphere formation, it may be possible to produce microspheres with the desired exposed peptides without the need for a separate conjugation step after the microspheres are formed. This would offer the advantage of

avoiding release of encapsulated agents during ligand conjugation, as the ligands would already be present as the microspheres are formed.

As with previous formulations of PLGA microspheres, PLGA/PEMA microspheres can encapsulate an essentially unlimited number of different agents. Release rates can be tailored to a specific application through adjustment of the lactide:glycolide monomer ratio in the PLGA polymer, without affecting the ability to conjugate ligands to the microsphere surface. The carboxylic acid groups incorporated into the microsphere surface provide a functional group capable of covalent coupling to a range of ligands. This microsphere formulation provides a biodegradable system with great flexibility in the design of targeted drug delivery vehicles, providing an opportunity to increase the efficiency of drug delivery in a variety of different applications.

REFERENCES

- [1] O'Hagan, D. T. *Advanced Drug Delivery Reviews* **1998**, *34*, 305-320.
- [2] Emerich, D. F.; Winn, S. R.; Snodgrass, P.; LaFreniere, D.; Agostino, M.; Wiens, T.; Xiong, H.; Bartus, R. T. *Pharmaceutical Research* **2000**, *17*, 776-781.
- [3] Kompella, U. B.; Bandi, N.; Ayalasomayajula, S. P. *Investigative Ophthalmology and Visual Science* **2003**, *44*, 1192-1201.
- [4] Eniola, A. O.; Hammer, D. A. *Journal of Controlled Release* **2003**, *87*, 15-22.
- [5] Mehta, R. C.; Jeyanthi, R.; Calis, S.; Thanoo, B. C.; Burton, K. W.; DeLuca, P. P. *Journal of Controlled Release* **1994**, *29*, 375-384.
- [6] Emerich, D. F.; Snodgrass, P.; LaFreniere, D.; Dean, R. L.; Salzberg, H.; Marsh, J.; Perdomo, B.; Arastu, M.; Winn, S. R.; Bartus, R. T. *Pharmaceutical Research* **2002**, *19*, 1052-1060.
- [7] Vasir, J. K.; Tambwekar, K.; Garg, S. *International Journal of Pharmaceutics* **2003**, *255*, 13-32.
- [8] Jani, P.; Halbert, G. W.; Langridge, J.; Florence, A. T. *Journal of Pharmacy and Pharmacology* **1989**, *41*, 809-812.
- [9] Andrianov, A. K.; Payne, L. G. *Advanced Drug Delivery Reviews* **1998**, *34*, 155-170.
- [10] Norris, D. A.; Sinko, P. J. *Journal of Applied Polymer Science* **1997**, *63*, 1481-1492.
- [11] Eldridge, J. H.; Hammond, C. J.; Meulbroek, J. A.; Staas, J. K.; Gilley, R. M.; Tice, T. R. *Journal of Controlled Release* **1990**, *11*, 205-214.
- [12] Jepson, M. A.; Clark, M. A.; Foster, N.; Mason, C. M.; Bennett, M. K.; Simmons, N. L.; Hirst, B. H. *Journal of Anatomy* **1996**, *189*, 507-516.

- [13] Tobio, M.; Gref, R.; Sanchez, A.; Langer, R.; Alonso, M. J. *Pharmaceutical Research* **1998**, *15*, 270-275.
- [14] Gref, R.; Minamitake, Y.; Peracchia, M. T.; Trubetskoy, V.; Torchilin, V.; Langer, R. *Science* **1994**, *263*, 1600-1603.
- [15] Peracchia, M. T.; Fattal, E.; Desmaële, D.; Besnard, M.; Noël, J. P.; Gomis, J. M.; Appel, M.; d'Angelo, J.; Couvreur, P. *Journal of Controlled Release* **1999**, *60*, 121-128.
- [16] Stella, B.; Arpicco, S.; Peracchia, M. T.; Desmaële, D.; Hoebeke, J.; Renoir, M.; D'Angelo, J.; Cattel, L.; Couvreur, P. *Journal of Pharmaceutical Sciences* **2000**, *89*, 1452-1464.
- [17] Ertl, B.; Heigl, F.; Wirth, M.; Gabor, F. *Journal of Drug Targeting* **2000**, *8*, 173-184.
- [18] Irache, J. M.; Durrer, C.; Duchêne, D.; Ponchel, G. *Pharmaceutical Research* **1996**, *13*, 1716-1719.
- [19] Eniola, A. O.; Willcox, P. J.; Hammer, D. A. *Biophysical Journal* **2003**, *85*, 2720-2731.
- [20] Eniola, A. O.; Rodgers, S. D.; Hammer, D. A. *Biomaterials* **2002**, *23*, 2167-2177.
- [21] Gebert, A.; Rothkotter, H.-J.; Pabst, R. *International Review of Cytology* **1996**, *167*, 91-159.
- [22] Neutra, M. R.; Giannasca, P. J.; Giannasca, K. T.; Kraehenbuhl, J.-P. *M cells and microbial pathogens*; Blaser, M. J., Smith, P. D., Ravdin, J. I., Greenberg, H. B. and Guerrant, R. L., Ed.; Raven Press Ltd.: New York, 1995, pp 163-178.
- [23] Sicinski, P.; Rowinski, J.; Warchol, J. B.; Jarzabek, Z.; Gut, W.; Szczygiel, B.; Bielecki, K.; Koch, G. *Gastroenterology* **1990**, *98*, 56-58.

- [24] Frey, A.; Neutra, M. R. *Targeting of mucosal vaccines to Peyer's patch M cells*; vonSpecht, B.-U., Ed.; Eukerdruck Marburg: Munich, 1997, pp 376-389.
- [25] Maloy, K. J.; Donachie, A. M.; O'Hagan, D. T.; Mowat, A. M. *Immunology* **1994**, *81*, 661-667.
- [26] Tabata, Y.; Inoue, Y.; Ikada, Y. *Vaccine* **1996**, *14*, 1677-1685.
- [27] Challacombe, S. J.; Rahman, D.; O'Hagan, D. T. *Vaccine* **1997**, *15*, 169-175.
- [28] Challacombe, S. J.; Rahman, D.; Jeffery, H.; Davis, S. S.; O'Hagan, D. T. *Immunology* **1992**, *76*, 164-168.
- [29] Chattaraj, S. C.; Rathinavelu, A.; Das, S. K. *Journal of Controlled Release* **1999**, *58*, 223-232.
- [30] Jones, D. H.; McBride, B. W.; Thornton, C.; O'Hagan, D. T.; Robinson, A.; Farrar, G. H. *Infection and Immunity* **1996**, *64*, 489-494.
- [31] Conway, M. A.; Madrigal-Estebas, L.; McClean, S.; Brayden, D. J.; Mills, K. H. G. *Vaccine* **2001**, *19*, 1940-1950.
- [32] Eyles, J. E.; Spiers, I. D.; Williamson, E. D.; Alpar, H. O. *Vaccine* **1998**, *16*, 2000-2009.
- [33] Whittum-Hudson, J. A.; An, L.-L.; Saltzman, W. M.; Prendergast, R. A.; MacDonald, A. B. *Nature Medicine* **1996**, *2*, 1116-1121.
- [34] Brayden, D. J. *European Journal of Pharmaceutical Sciences* **2001**, *14*, 183-189.
- [35] Tacket, C. O.; Reid, R. H.; Boedeker, E. C.; Losonsky, G.; Nataro, J. P.; Bhagat, H.; Edelman, R. *Vaccine* **1994**, *12*, 1270-1274.
- [36] Clark, M. A.; Jepson, M. A.; Simmons, N. L.; Booth, T. A.; Hirst, B. H. *Journal of Histochemistry and Cytochemistry* **1993**, *41*, 1679-1687.
- [37] Chen, H.; Torchilin, V.; Langer, R. *Pharmaceutical Research* **1996**, *13*, 1378-1383.

- [38] Clark, M. A.; Blair, H.; Liang, L.; Brey, R. N.; Brayden, D.; Hirst, B. H. *Vaccine* **2001**, *20*, 208-217.
- [39] Foster, N.; Clark, M. A.; Jepson, M. A.; Hirst, B. H. *Vaccine* **1998**, *16*, 536-541.
- [40] Giannasca, P. J.; Giannasca, K. T.; Leichtner, A. M.; Neutra, M. R. *Infection and Immunity* **1999**, *67*, 946-953.
- [41] Porta, C.; James, P. S.; Phillips, A. D.; Savidge, T. C.; Smith, M. W.; Cremaschi, D. *Experimental Physiology* **1992**, *77*, 929-932.
- [42] Weltzin, R.; Lucia-Jandris, P.; Michetti, P.; Fields, B. N.; Kraehenbuhl, J. P.; Neutra, M. R. *The Journal of Cell Biology* **1989**, *108*, 1673-1685.
- [43] Gabor, F.; Wirth, M.; Jurkovich, B.; Haberl, I.; Theyer, G.; Walcher, G.; Hamilton, G. *Journal of Controlled Release* **1997**, *49*, 27-37.
- [44] Underdown, B. J.; Dorrington, K. J. *Journal of Immunology* **1974**, *112*, 949-959.
- [45] Hussain, N.; Florence, A. T. *Pharmaceutical Research* **1998**, *15*, 153-156.
- [46] Forrest, B. D. *Advances in Experimental Medicine and Biology* **1995**, *371B*, 1649-1652.
- [47] Lavelle, E. C.; Grant, G.; Pusztai, A.; Pfuller, U.; O'Hagan, D. T. *Immunology* **2000**, *99*, 30-37.
- [48] Liley, P. E.; Thomson, G. H.; Friend, D. G.; Daubert, T. E.; Buck, E. *Physical and Chemical Data*; 7 ed.; Perry, R. H., Green, D. W. and Maloney, J. O., Ed.; McGraw-Hill: New York, 1997, pp 2-1 - 2-374.
- [49] Ertl, B.; Platzer, P.; Wirth, M.; Gabor, F. *Journal of Controlled Release* **1999**, *61*, 305-317.
- [50] Scholes, P. D.; Coombes, A. G. A.; Illum, L.; Davis, S. S.; Watts, J. F.; Ustariz, C.; Vert, M.; Davies, M. C. *Journal of Controlled Release* **1999**, *59*, 261-278.
- [51] Montisci, M.-J.; Giovannuci, G.; Duchêne, D.; Ponchel, G. *International Journal of Pharmaceutics* **2001**, *215*, 153-161.

- [52] Gref, R.; Couvreur, P.; Barratt, G.; Mysiakine, E. *Biomaterials* **2003**, *224*, 4529-4537.
- [53] Müller, M.; Vörös, J.; Csúcs, G.; Walter, E.; Danuser, G.; Merkle, H. P.; Spencer, N. D.; Textor, M. *Journal of Biomedical Materials Research* **2003**, *66A*, 55-61.
- [54] Pappo, J.; Ermak, T. H.; Steger, H. J. *Immunology* **1991**, *73*, 277-280.
- [55] Carreno-Gomez, B.; Woodley, J. F.; Florence, A. T. *International Journal of Pharmaceutics* **1999**, *183*, 7-11.
- [56] Hussain, N.; Jani, P. U.; Florence, A. T. *Pharmaceutical Research* **1997**, *14*, 613-618.
- [57] Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: New York, 1996.
- [58] O'Donnell, P. B.; McGinity, J. W. *Advanced Drug Delivery Reviews* **1997**, *28*, 25-42.
- [59] Kerneis, S.; Bogdanova, A.; Kraehenbuhl, J.-P.; Pringault, E. *Science* **1997**, *277*, 949-952.
- [60] Kerneis, S.; Caliot, E.; Stubbe, H.; Bogdanova, A.; Kraehenbuhl, J.-P.; Pringault, E. *Microbes and Infection* **2000**, *2*, 1119-1124.
- [61] Liang, E.; Kabcenell, A. K.; Coleman, J. R.; Robson, J.; Ruffles, R.; Yazdanian, M. *Journal of Pharmacological and Toxicological Methods* **2002**, *46*, 93-101.
- [62] El Bahi, S.; Caliot, E.; Bens, M.; Bogdanova, A.; Kerneis, S.; Kahn, A.; Vandewalle, A.; Pringault, E. *Journal of Immunology* **2002**, *168*, 3713-3720.
- [63] Debard, N.; Sierro, F.; Kraehenbuhl, J.-P. *Seminars in Immunology* **1999**, *11*, 183-191.
- [64] van der Lubben, I. M.; van Opdorp, F. A. C.; Hengeveld, M. R.; Onderwater, J. J. M.; Koerten, H. J.; Verhoef, J. C.; Borchard, G.; Junginger, H. E. *Journal of Drug Targeting* **2002**, *10*, 449-456.

- [65] Tyrer, P.; Foxwell, A. R.; Kyd, J.; Harvey, M.; Sizer, P.; Cripps, A. *Biochemical and Biophysical Research Communications* **2002**, *299*, 377-383.
- [66] Gullberg, E.; Leonard, M.; Karlsson, J.; Hopkins, A. M.; Brayden, D.; Baird, A. W.; Artursson, P. *Biochemical and Biophysical Research Communications* **2000**, *279*, 808-813.
- [67] Neutra, M. R.; Mantis, N. J.; Frey, A.; Giannasca, P. J. *Seminars in Immunology* **1999**, *11*, 171-181.
- [68] Clark, M. A.; Jepson, M. A.; Simmons, N. L.; Hirst, B. H. *Cell & Tissue Research* **1995**, *282*, 455-461.
- [69] McKisic, M. D.; Paturzo, F. X.; Smith, A. L. *Transplantation* **1996**, *61*, 292-299.
- [70] Ebel, J. P. *Pharmaceutical Research* **1990**, *7*, 848-851.
- [71] Kuo, S. C.; Lauffenburger, D. A. *Biophysical Journal* **1993**, *65*, 2191-2200.
- [72] Brunk, D. K.; Hammer, D. A. *Biophysical Journal* **1997**, *72*, 2820-2833.
- [73] Mahoney, M. J.; Saltzman, W. M. *Nature Biotechnology* **2001**, *19*, 934-939.
- [74] Ayhan, H.; Gurhan, I.; Piskin, E. *Artificial Cells, Blood Substitutes, and Immobilization Biotechnology* **2000**, *28*, 155-171.
- [75] Herrmann, J.; Bodmeier, R. *Journal of Controlled Release* **1995**, *36*, 63-71.
- [76] Dai, W.; Belt, J.; Saltzman, W. M. *Bio/Technology* **1994**, *12*, 797-801.