Controlled Release of Human Immunoglobulin G. 2. Morphological Characterization

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Abstract □ Human immunoglobulin G (IgG) serves as an important chemotherapeutic agent for a number of immunological ailments and as a carrier in the targeted delivery of other therapeutic agents. This paper deals with the characterization of IgG-dispersed monolithic matrixes of different geometries, prepared using a nonbiodegradable polymer carrier EVAc. The morphological changes associated with the matrix during drug release was studied using scanning electron microscopy, polarizing microscopy, atomic force microscopy, and X-ray photoelectron microscopy, and the results were compared. The study answered the burst effect problem significantly and illustrated the potential of these techniques in understanding the morphological structure of matrixes and mode of release kinetics.

Introduction

The topic of controlled drug release has received significant contributions from the scientific world ever since the work of Langer and Folkman in the mid-1970s.[1] A number of different types of polymers[2−6] have been used for the controlled release studies, and the mode of release varies with the polymer type. Although release kinetics studies and the fitting of experimental data to model equations would give us some understanding of the type of release mechanism involved, the results obtained can be ascertained by conducting matrix characterization studies for matrixes during different stages of drug release. Release kinetics of immunoglobulin G (IgG), from a nonbiodegradable EVAc polymer, have been discussed elsewhere.[7] It was noted that dissolution plays a significant role during the initial stages of release, while diffusion predominates in the later period. This variation in the release mechanism necessitated the use of combination of models to describe the release behavior. In this report, an extensive study has been made in the morphological characterization of these matrixes both in the qualitative and quantitative sense. A variety of techniques such as the scanning electron microscopy, atomic force microscopy, polarizing microscopy, and X-ray photoelectron microscopy were used for matrix characterization.

Scanning electron microscopy (SEM) still serves as a preliminary surface characterization technique for numerous drug delivery systems.[3,8−10] The technique is greatly appreciated when applied to biodegradable systems in which marked changes in matrix morphology occur with release. However, in the present study, nonbiodegradable EVAc matrixes of different geometries under varied stages of release were examined under SEM for both the surface and cross-section analysis.

Atomic force microscopy (AFM) is used to study the surface features of polymeric materials by visualizing their morphology, nanostructure, molecular order, and surface compositional mapping in heterogeneous samples with a resolution down to the atomic level.[11] The need to choose AFM for matrix characterization is because of its attractive feature of obtaining two- and three-dimensional topographical features of material surfaces at extremely high resolution in either vacuum, liquid, or ambient atmospheres. AFM is a mechano-optical instrument, which detects atomic-level forces ($\sim nN$) through optical measurements of movements of a very sensitive cantilever tipped with a hard, pyramid-shaped crystal that moves along surfaces. Typical working mode of AFM is the contact mode, where the AFM tip remains (more or less) in contact with the surface (some friction may be observed). Most AFMs have vertical resolution below 0.1 nm range, whereas lateral resolution is usually lower due to the sharp tip and the small loading force involved.

Polarizing microscopy (PM), when equipped with a hot stage, is a valuable tool for measuring phase transitions and crystallization kinetics. When the sample is placed between two crossed polarizing filters, different colors based on varying thickness, texture, stress level, and bond arrangement of polymer chains can be observed. The first polarizing filter polarizes or limits the vibration of light waves to one plane. When the polarized light passes through the polymer, its direction of polarization is altered depending on the thickness and texture of the polymer. The final direction of polarization determines whether light of a certain wavelength can pass through the second polarizing filter. The importance of this technique lies in the fact that it is able to qualitatively analyze the change in IgG concentration on the EVAc matrix surface.

X-ray photoelectron microscopy (XPS) is by far the most widely used surface spectroscopic instrument. When a surface is irradiated with soft X-ray photons, electrons from inner shells can be ejected with a definite binding energy. Therefore, XPS is capable of elemental analysis since no two atoms of the periodic table exhibit the same set of binding energies. Qualitative analysis of the elements can be easily performed by wide scans, in which all the available energy range is explored. Quantitative elemental analysis is performed by detail scans, where a small portion of the binding energy range is acquired with a higher spectral resolution than in the wide scans.

The characterization studies were performed for matrixes in different stages of drug release. The results obtained were then used to get a reasonable explanation for the type of release mechanism and the extent of drug distribution in the matrix.

Materials and Methods

IgG-EVAc systems of different geometries, viz. slabs, microspheres, and cubes, were fabricated as described in a previous

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Table 1—Mean Diameter of Microspheres of Different % IgG Loadings

microsphere type	mean diameter[a] (mm)	standard deviation
10% IgG loading	1.09	0.082
20% IgG loading	1.19	0.065
30% IgG loading	1.20	0.101

[a] Average of 20 beads.

paper.[7] Blank matrixes (without IgG) were also fabricated in a similar manner. The different matrixes were then subjected to release in 0.1 M phosphate buffer, pH 7.4, for a time span of 3 to 8 weeks. They were then removed by forceps, blotted dry by tissue, and subjected to characterization studies.

Scanning Electron Microscopy (SEM)—Samples of slab and microsphere were stuck on to metallic cylinders by making use of a double-sided carbon adhesive tape, which were then sputter-coated using an ion sputtering device (JFC-1100E, JEOL Co., Tokyo, Japan) and observed under SEM (JSM-T330A, JEOL Technics Co. Ltd., Tokyo, Japan).

Atomic Force Microscopy (AFM)—Samples of slab were cut into thin sections using a surgical blade (Hecos, Shanghai, China) and observed under AFM (Topometrix, TMX-2000, Topometrix Corp., Santa Clara, CA). Explorer AFM was used as the stage type and the image resolution was fixed at 200 × 200 pixels under forward scan direction and 0° rotation with system calibration. In addition, this instrument employs a "double cross" cantilever, which restricts the motion of the tip to the "Z" direction (normal to the sample surface). Scanning was done using an E449701 type scanner at the rate 200 μm/s, and the sample bias was fixed at 1 mV. Microspheres, because of their natural curvature, are difficult to be observed under AFM and hence are not shown.

Polarizing Microscopy (PM)—Samples of slab and microsphere were observed under PM-BX50 (Olympus, Japan). A 530 nm sensitive tint plate (U-TP530) was used as a test plate compensator, which resulted in a magenta background for the pictures taken. A hot stage (Cole-Parmer Digital Hot Plate, Model no. 8207C-2 Vernon Hills, IL) was used to increase the specimen temperature at selected rates, and the system was capable of taking a time series of photographs while holding the sample at a constant temperature. Visual observations of specimen transitions and the temperatures at which they occur may be recorded.

X-ray Photoelectron Spectroscopy (XPS)—Samples of slab were observed under XPS (VG ESCALAB MKII spectrometer) with Mg Kα (1253.6 eV, 120W) as X-ray source and neutral C1s (284.6 eV) as BEs reference to determine the elemental composition of the matrix. The take-off angle was set at 75°, and the chamber pressure was maintained at $\leq 1 \times 10^{-8}$ mbar. FWHM was kept constant for all the components. To investigate the distribution of IgG on the surface of IgG/EVAc system, a combined XPS method of wide scan with detail scan was employed. Elemental ratios of O/C and N/C were calculated by the method of detail scan. For the purpose of comparison with 30% IgG film sample, we also grinded it into fine powder using a mortar and pestle and then measured its XPS value.

Results and Discussion

(a) Particle Size Analysis—In the present study, the dependence of microsphere size on the needle size and the drug loading (which affects the viscosity and surface tension of the solution) was examined by maintaining a uniform extrusion rate (2−3 mL/min). The microsphere particle size was measured using the Nikon SMZ-1 microscope. The size distribution was compared for spheres with different drug loadings and for spheres with different diameters prepared using different sized needles. The results are tabulated in Tables 1 and 2. It is quite clear that the size increases as drug loading is increased and as needle size is increased. In spite of the size being in the range of 1000 μm, we can still consider these beads as microspheres since their size is within the range ca. 50 nm−2 mm.[12]

(b) SEM—Observations of the SEM pictures for slabs (Figure 1) show that the EVAc−IgG unreleased (Figure 1a)

Table 2—Mean Diameter of Microspheres Prepared Using Different Needle Sizes for the Blank (without IgG) and the 20% IgG-Loaded Cases

	mean diameter[a] (mm)		standard deviation	
needle size	blank	20% loading	blank	20% loading
16 gauge	0.99	1.19	0.071	0.065
19 gauge	0.88	0.95	0.097	0.062
23 gauge	0.68	0.81	0.096	0.049

[a] Average of 20 beads.

matrix surface was uniform without any clear demarcation of the IgG molecules in the matrix. The matrix surface, which can be described as an extended, highly loose globular, viscous, cloudy surface was found to be spread uniformly over and within the matrix (Figure 1b). This could not be seen in the blank sample of EVAc polymer (no drug case) matrix, which was made up of a clear continuous phase. Even the cross-section of the blank showed a clear phase distribution without the presence of any matrix deformities or pores. In the case of EVAc−IgG matrix subjected to release for 45 days (Figure 1c), the deformation of the continuous polymer phase can be clearly observed. The deformation might be due to the swelling of the dispersed IgG particles upon uptake of diffusant.[13] Further, cracks and channels, possibly due to the interconnection of the pores were also observed in the cross-section of Figure 1d. Figures 1e−h are the SEM pictures of 30% loaded cubic samples. Compared with the slab samples, the unreleased sample (Figure 1e) exhibits a similar continuous undeformed phase, whereas the cross-section (Figure 1f) reveals the presence of "pocket-filled IgG cluster" like structures. Surface (Figure 1g) and cross-section (Figure 1h) of the sample subjected to release for two months reveal deformities as expected. However, the cross-section reveals distinct pores rather than channels (seen in Figure 1d), which can be attributed to the difference in geometry between the two cases.

SEM pictures of microsphere are shown in Figure 2. The thready polymer entanglements are clearly visible in Figure 2a. Figure 2b shows a portion of the blank sample cut off. It was noticed that the cross-section of the blank exhibits a hollow region. This feature might be due to the evaporation of methylene chloride from the matrix during its fabrication. The 30% loaded sample in Figure 2c shows certain complications in the surface morphology. Again, the cross-section in Figure 2d of the loaded sample before release shows a hollow region. However, no pores are visible on the matrix surface. The sample subjected to release for three weeks in Figure 2e shows a little bit of swelling, and minute pores or channels can be noticed (possibly due to the relaxation of the polymer chains). Finally, Figure 2f, a cross-section of the loaded sample taken in the transverse direction, after three weeks of release, reveals pores along with the expected hollow region observed along the side. Hence, a common feature noted in all the three geometries is the increase of matrix porosity with prolonged exposure to the release medium. Since the polymer is hydrophobic and nonbiodegradable, the occurrence of such deformities such as pores and cracks can be attributed due to the drug−solvent interaction—dissolution of the drug in the solvent, swelling of the drug due to solvent uptake, and gradual diffusion through the solvent-filled pores and channels.

(c) AFM—AFM pictures of the slab samples are shown in Figure 3. Observation of the blank sample (Figure 3a) shows a smooth, flat surface. The numerous tiny spots, which appear on the figure, are not pores but are rather microscopic depressions on the surface due to the evaporation of the solvent. On observing the IgG-loaded sample

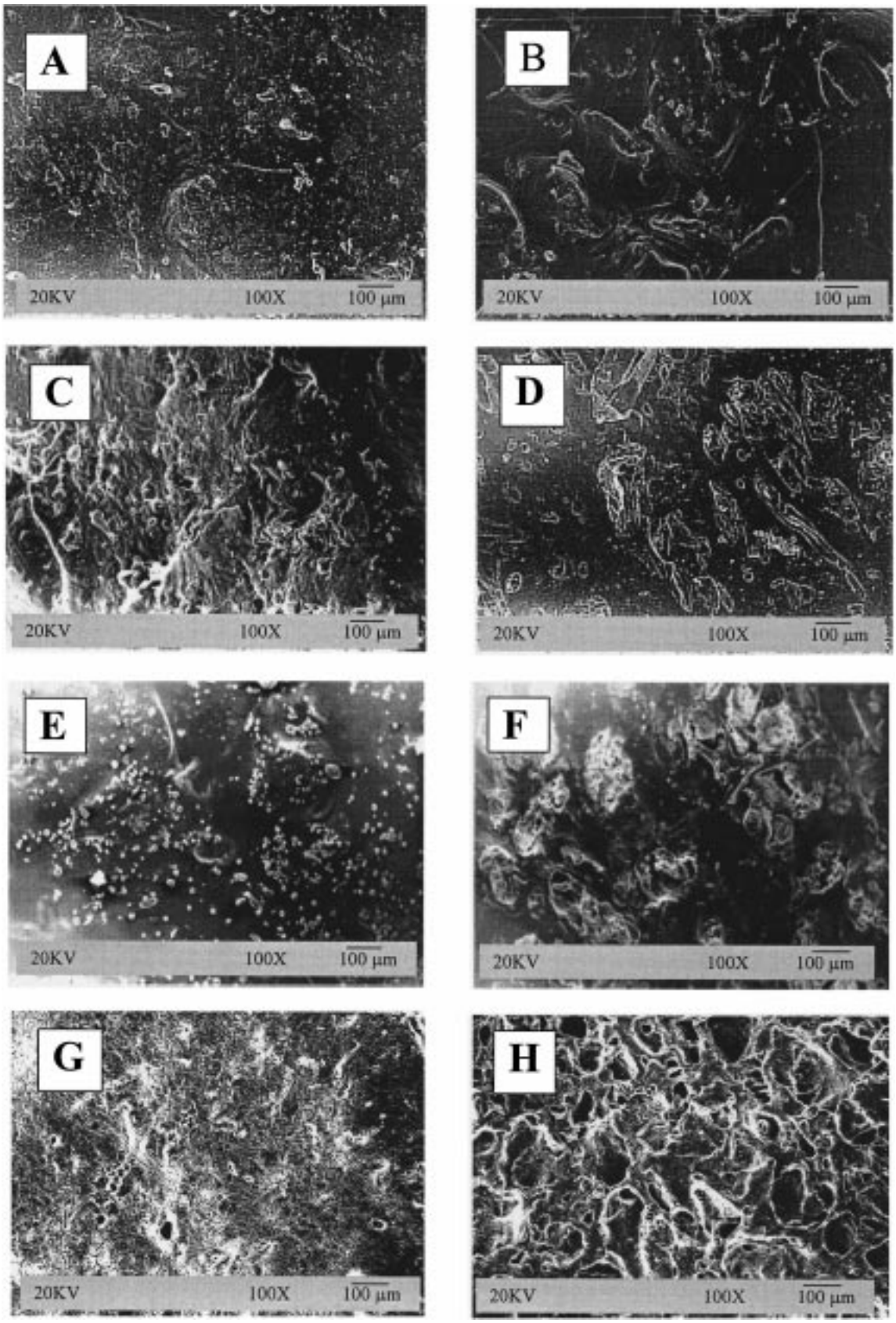

Figure 1—Scanning electron microscopy photographs of 30% IgG loading for (a) slab, before release, (b) slab cross-section, before release, (c) slab, after 45 days of release, (d) slab cross-section, after 45 days release, (e) cube, before release, (f) cube cross-section, before release, (g) cube, after 2 months of release, (h) cube cross-section, after 2 months of release. All pictures are of 200× magnification.

before release (Figure 3b), we find that the surface has a fluffy appearance with numerous bumps and undulations. The depressions seen on the blank sample have been completely filled up, and a homogeneous matrix surface could be observed. The dark spot appearing on the far corner of the figure is just an artifact created due to the

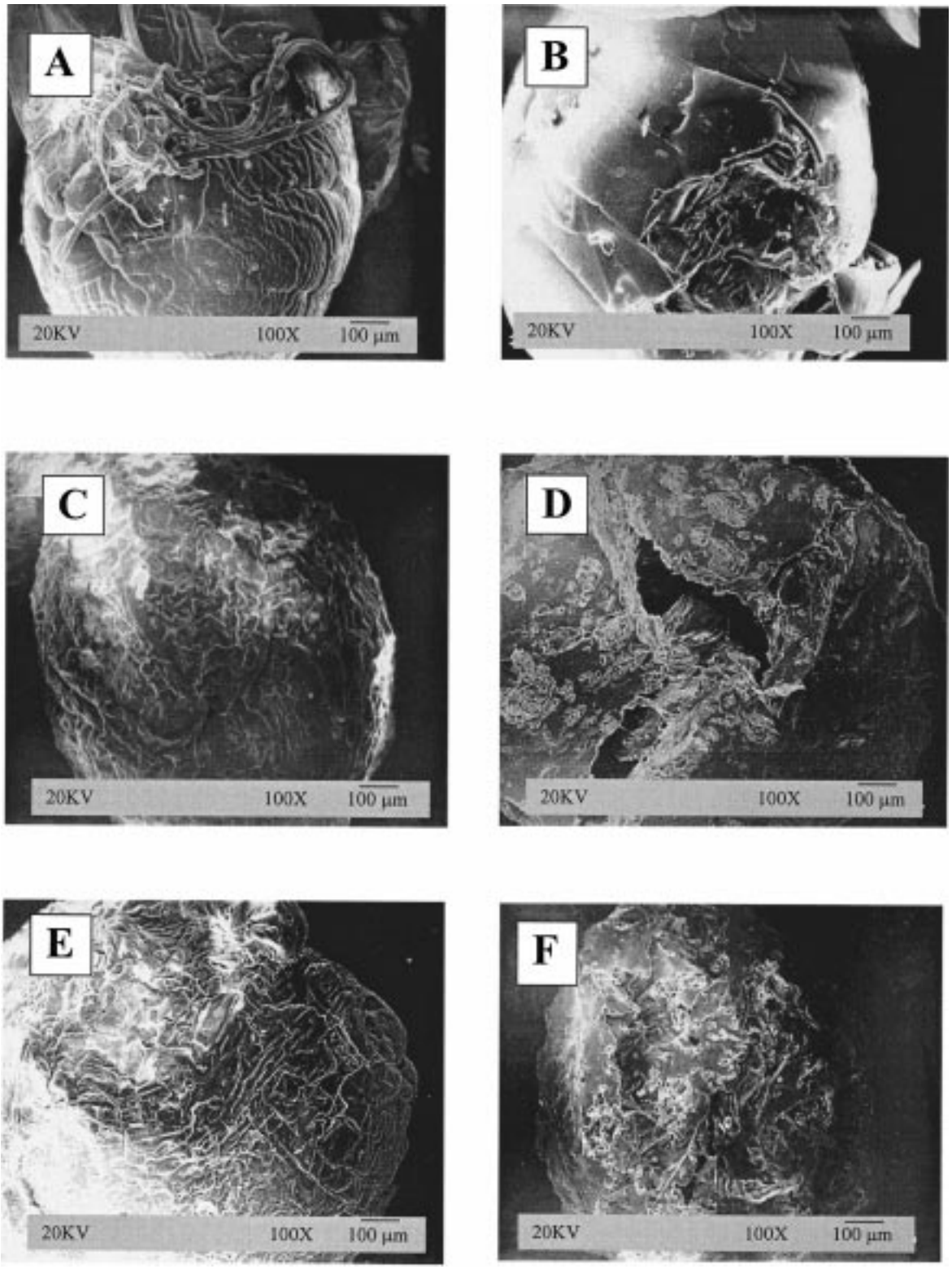

Figure 2—Scanning electron microscopy photographs of microspheres for (a) blank, (b) cross-section of blank, (c) 30% IgG loading, before release, (d) cross-section of 30% IgG loading, before release, (e) 30% IgG loading, after 3 weeks of release, (f) cross-section of 30% IgG loading, after 3 weeks release. All pictures are of 100× magnification.

sectioning of the sample for AFM studies. Figure 3c is an AFM picture of the sample subjected to release for 30 days. We can clearly see pores on the surface, with occasional strips of fluffy mass seen in the before release sample. AFM results confirm the nonbiodegradable nature of the EVAc polymer and backs the SEM results of drug dissolution into the buffer-filled pores followed by diffusion.

(d) PM—The PM pictures of the slab can be viewed in Figures 4a–c. The blank sample can be observed in Figure 4a. The sample placed between two crossed polarizing filters gives different shades of colors depending on the depths at which the light penetrates, and the colors are reflected based on the refractive index of the component. The complexity of the structure increases with the drug loading as shown in the 30% drug-loaded sample before release (Figure 4b). Numerous opaque dark spots could be

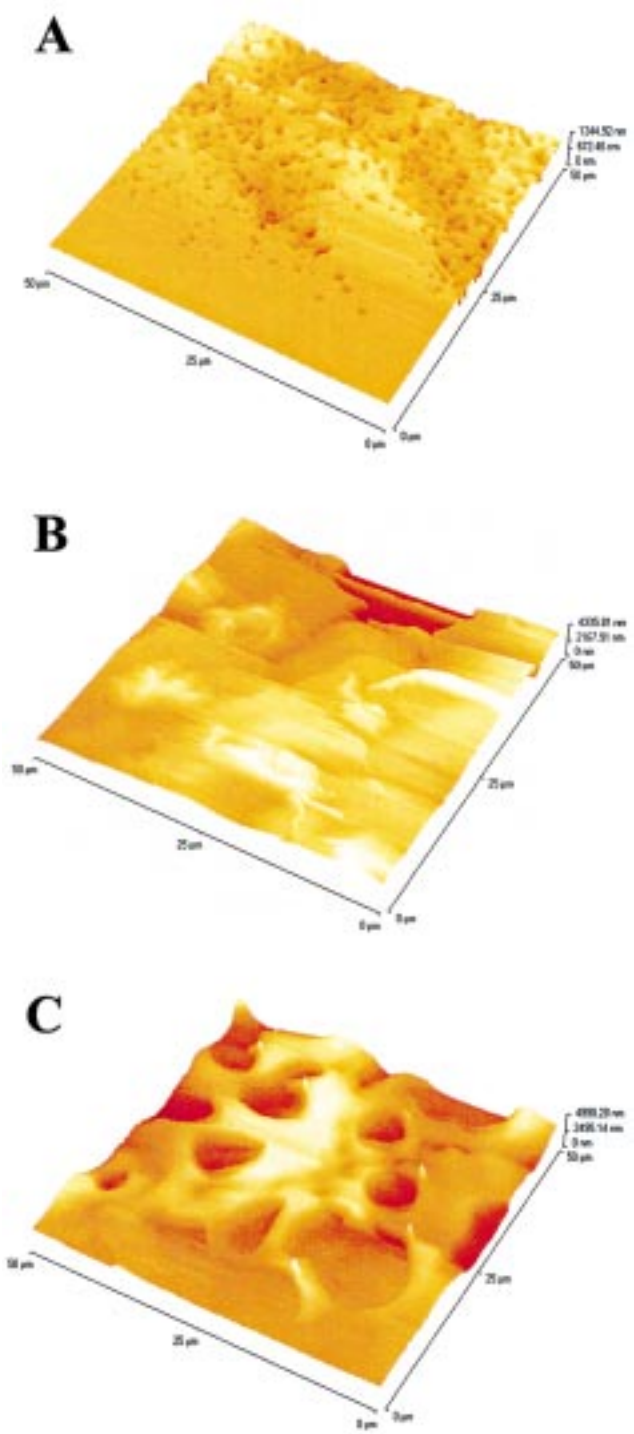

Figure 3—Atomic force microscopy pictures of slabs for (a) blank, (b) 30% IgG loading, before release, (c) 30% IgG loading, after 30 days release.

observed which are not present in the blank sample. The fact that these are IgG clusters cannot be completely ignored. This assumption is further strengthened by the reduction of such a structural feature in the sample subjected to release for 45 days, as shown in Figure 4c. A thorough analysis of the dark spots on Figure 4b was done by magnifying them and comparing with the PM pictures of raw IgG molecules and they did show similar structure (Figure 5). PM pictures of microspheres can be viewed in Figures 4d–f. The blank sample in Figure 4d shows some similarity with the SEM picture in Figure 2a with respect to the external morphology. In fact, higher magnification of Figure 4d reveals the presence of some polygonal structures on the matrix surface, which could also be observed in the SEM at higher magnifications. Similar to Figure 4b, dark spots can be observed in the 30% drug-loaded sample before release (Figure 4e) which are likely to be IgG molecules, but the decreased concentration of these spots when compared to the slab surface (Figure 4b) is attributed to the dosage form involved. The reduced black spot concentration on the microsphere surface also answers the decrease in burst effect observed for this geometry when compared to the case of slab geometry. The black spot concentration decreases with the release of IgG molecules, as shown in the sample subjected to release for 3 weeks (Figure 4f). However, this decrease is not as pronounced with respect to the relative decrease in concentration of black spots from Figure 4b to 4c. This is quite logical looking at the fact that only about 55% of IgG has been released after 3 weeks for a microsphere when compared to the 81% release observed for the slab after 46 days.[7]

(e) XPS—The wide scan and detail scan XPS pictures are shown in Figure 6. Theoretically, the photoelectron binding energy E_b is given by the following relation[14]

$$E_b = h\nu - E_k - \phi \qquad (1)$$

where $h\nu$ is the photon energy of the X-ray source (1253.6 eV in the present case), E_k is the kinetic energy of the photoelectron, and ϕ is the spectrometer work function (defined as the energy required to remove the uppermost electrons).

Examination of blank sample by wide scan XPS showed that the main elements of EVAc were C (carbon) and O (oxygen), without N (nitrogen), as seen in Figure 6a, while the wide scan XPS of pure IgG showed the presence of a N1s peak along with C1s and O1s peaks, thereby indicating the presence of nitrogen in pure IgG (Figure 6d). The peak of N1s is centered near 399.0 eV (Table 3), a value characteristic of the amino group from a protein. Hence, we may assume the concentration of nitrogen determined by the XPS method as an indirect measure of IgG concentration on the surface of the IgG–EVAc system. Interestingly, 30% IgG-loaded samples, exhibited a stronger N1s peak for its grinded version (Figure 6b) than its film version (Figure 6c). Further, detail scans of XPS revealed that the powdered sample had a higher N/C ratio than the film sample (Table 3). The reduced surface concentration of IgG in the film sample can be due to the relatively faster migration of EVAc to the surface compared to IgG during the evaporation of methylene chloride, which was ultimately reflected in the results due to the surface specific detection property of XPS. On the other hand, the sample obtained by grinding the film to a fine homogeneous blend of powder, exhibited an increased concentration of IgG on the surface, resulting in a stronger N1s peak in the wide scan XPS and a corresponding increase in the N/C ratio. Since XPS can be used to measure the elemental composition present on sample surfaces alone, we utilized this aspect to our advantage in trying to get an explanation for the "burst effect" or the increased release rate of drugs at the initial time period. Since most of the element nitrogen (N) is present in the IgG molecule (constituting about 85% wt in the Venoglobulin-I sample[15] used for the release study), an analysis of the N/C composition for the slab sample (top surface) and its grinded version (sample grinded to get a flat layer) will give the approximate IgG composition in the top surface of the slab and the overall IgG composition of the slab. The percentage of IgG present on the top surface calculated in this manner was found almost equal to the fraction of IgG released in the first hour (with an error less than 10%). Hence the burst effect might

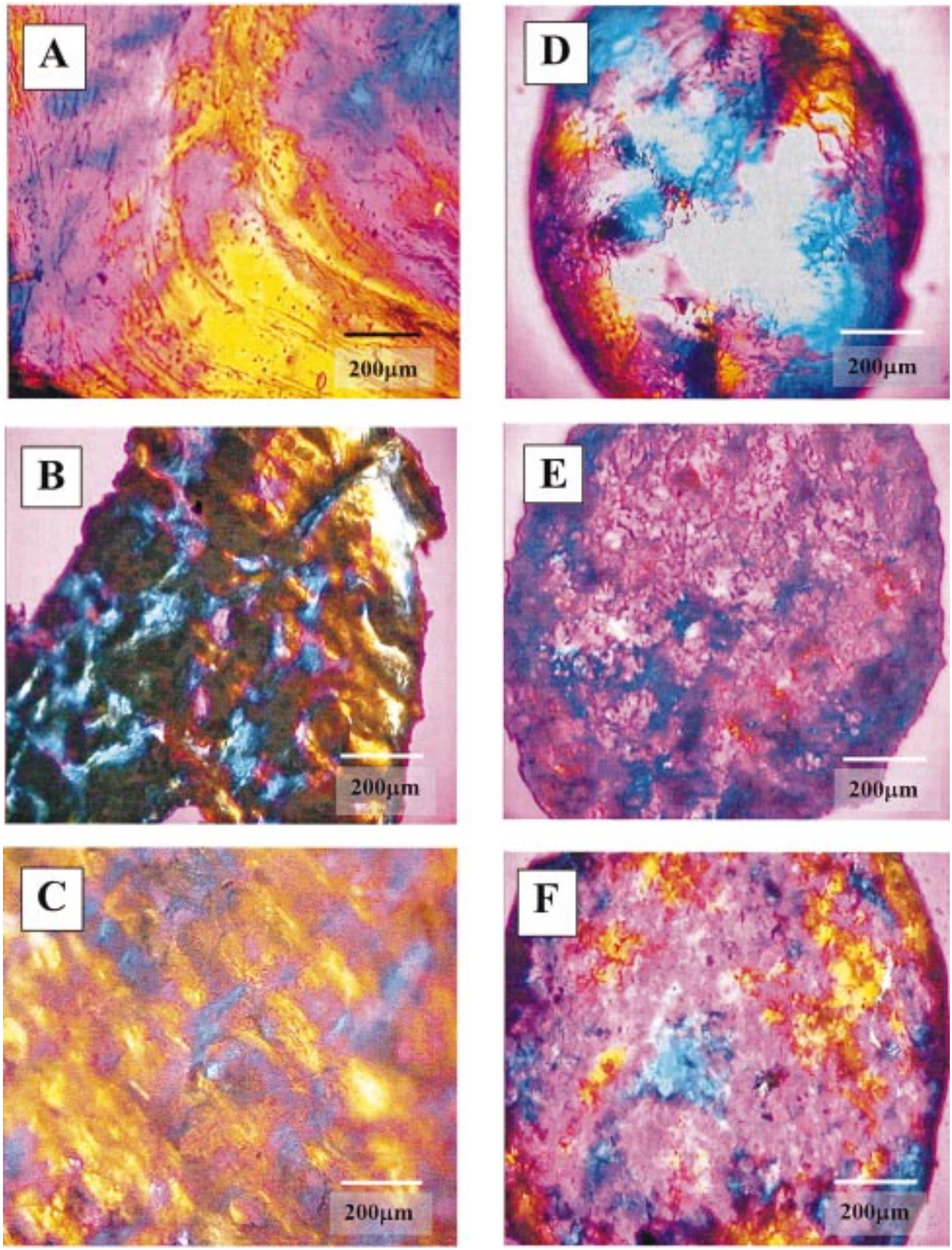

Figure 4—Polarizing microscopy pictures for (a) slab, blank, (b) slab, 30% IgG loading, before release, (c) slab, 30% IgG loading- after 45 days release, (d) microsphere, blank, (e) microsphere, 30% IgG loading, before release, (f) microsphere, 30% IgG loading, after 3 weeks of release.

be primarily due to the surface concentration of IgG molecules, and this can be avoided by the use of coated matrixes.

The utilization of microspheres of size range in the order of 800 to 1100 μm prevents phagocytosis by macrophages. Immunologically speaking, macrophages play an important

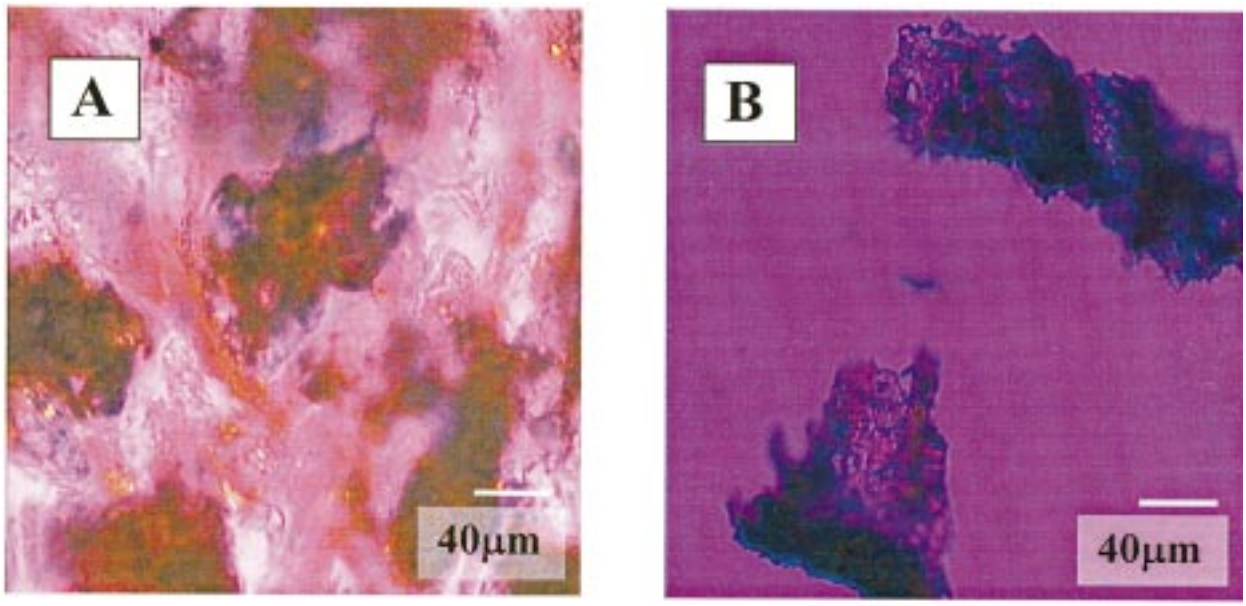

Figure 5—Polarizing microscopy pictures of (a) black spot on the matrix surface, (b) raw IgG clusters.

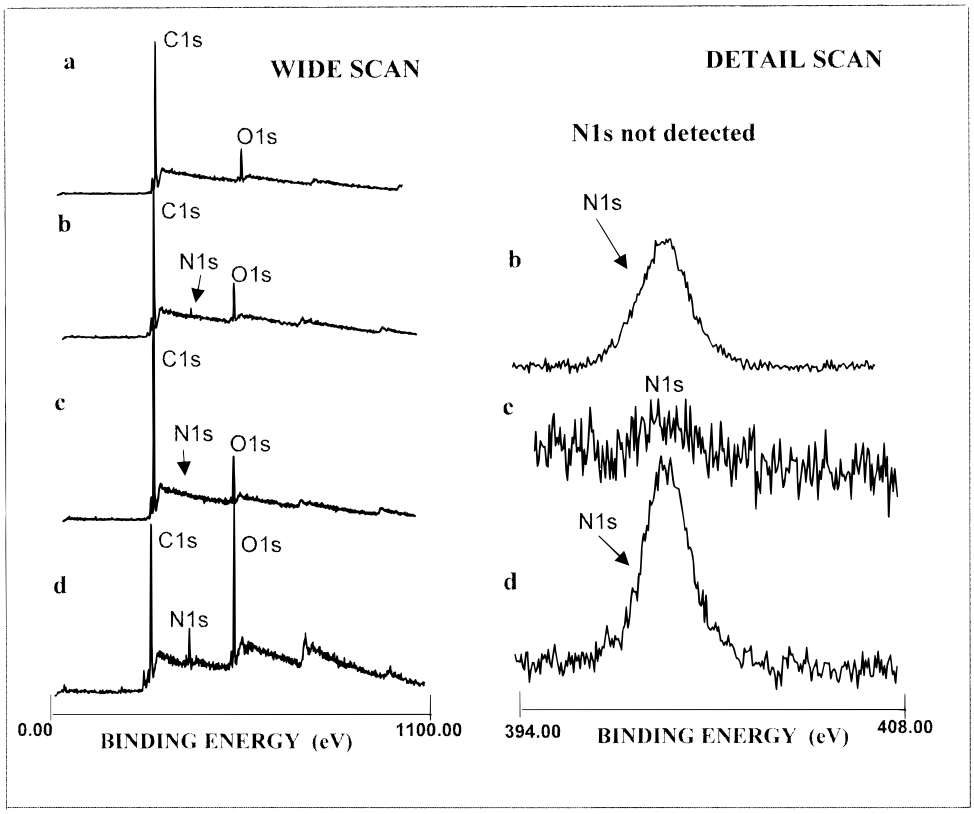

Figure 6—XPS spectrum for (a) blank, (b) 30% IgG loading, before release, powder, (c) 30% IgG loading, before release, film, (d) pure IgG.

Table 3—Detail Scan Data of XPS for Different Samples

sample	binding energy N1s (eV)	N/C	O/C
blank	–	–	0.1249
30% IgG-loaded film	399.10	0.0024	0.1323
30% IgG-loaded powder	399.52	0.0395	0.1799
IgG powder	400.00	0.1361	0.4328

role in the body's defense mechanism by engulfing or phagocytosing foreign bodies or antigenic particles and triggering the formation of antibodies to act against these antigens. Hence, microspheres of size range less than 800 μm have an increased probability of being engulfed by macrophages, which triggers the formation of anti-antibodies, thereby affecting the basic principle of treatment. This explains the role of microsphere size selection for therapeutic purposes.

Conclusions

Matrix characterization studies were performed using SEM, AFM, PM, and XPS. SEM and AFM studies gave a clear picture of the type of morphological changes encountered by the matrix in the release study. The results

indicate that the drug release mechanism is controlled by diffusion−dissolution irrespective of the matrix geometry. PM studies gave insight into the extent of IgG distribution in the polymer matrix while XPS results showed that the burst effect observed in the release studies during the initial period might be due to the surface concentration of IgG molecules. These characterization studies would therefore serve as supporting evidence to the in vitro release kinetics studies for IgG release through an EVAc backbone.

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