AAPS PharmSciTech, Vol. 16, No. 4, August 2015 (© 2015)
DOI: 10.1208/s12249-015-0292-2

Research Article

The Anti-Melanoma Efficiency of the Intratumoral Injection of Cucurbitacin-Loaded Sustained Release Carriers: *In Situ*-Forming Implants

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Received 16 October 2014; accepted 8 January 2015; published online 22 January 2015

Abstract. Our previous studies revealed that the PLGA-based particulate systems loaded with cucurbitacin showed limited anti-melanoma efficiency in xenograft animal models after intratumoral injection, which was due to the undesirable initial burst release and the leakage of the particulate carriers from the injection site through the pinhole. In this paper, two categories of *in situ*-forming implants (ISFIs) for intratumoral injection, PLGA ISFIs and SAIB ISFIs, were systemically evaluated for their potentials for on solid tumor treatment *via* intratumoral injection. The *in vitro* drug release profiles of these two ISFIs were different due to the different sol–gel transition properties. The pharmacodynamics results revealed that SAIB ISFIs displayed obvious therapeutic efficiencies to melanoma, and multi-points injection of SASIB ISFIs displayed better efficiency than single-point injection. The different sol–gel transition properties and mechanism for PLGA ISFIs and SAIB ISFIs affected both the drug release and strongly impacted the pharmacokinetic parameters and pharmacodynamic effectiveness. Also, the adhesive property of SAIB to the local tissue could extend the retention and inhibit the leakage of the SAIB ISFIs, thus enhanced the anticancer effectiveness. Comparison of the various intratumoral injection systems, appropriate drug release profiles (lower initial burst and steady release) and good retention (minimum leakage from the injection site) would benefit to the antitumor effects of the intratumoral depots.

KEY WORDS: anti-melanoma efficiency; cucurbitacin; drug release; *in situ*-forming implants (ISFIs); intratumoral injection.

INTRODUCTION

In our previous studies (1), cucurbitacin-loaded PLGA (poly(lactic/glycolic) acid) particles, a multi-particulate dispersed depot, were evaluated for its anti-melanoma efficiency. The results revealed that the initial burst release strongly impacted the anti-melanoma efficiency, and the extended drug retention in tumor could significantly reduce the side effects. However, due to the high internal pressure in tumors, which may force the particles to leak out through the pinhole after intratumoral injection, cucurbitacin-loaded PLGA particles failed to display satisfactory antitumor efficiency in the xenograft model. In this study, the anti-melanoma efficiency of intratumoral injection of novel and widely used sustained release carriers, *in situ*-forming implants (ISFIs), was evaluated.

An injectable ISFIs system is a miscible blend composed of a water-insoluble matrix and a water-miscible, biocompatible solvent (2–5). ISFIs can be injected as low-viscosity solutions. After the injection of the ISFIs into an aqueous tissue environment, the water-miscible solvent diffuses out of the system, and the matrix material then precipitates to form a solid or gel-like semi-solid depot *in vivo* (6,7). The embedded drug can release in a controlled fashion from the *in situ* formed depot (4,5). ISFIs formulations as the injectable depots, are therefore an option to avoid constant infusion or high frequent injection, and can minimize undesirable side effects caused by fluctuating plasma drug levels (8).

ISFIs systems can be formed using biodegradable polymers as the matrix material. For example, the PLGA ISFIs systems have been intensively investigated in the pre-clinical, clinical, and postmarket stages, especially for local anesthesia/analgesia or local anti-inflammatory treatment (9–12). The Atrigel® technology introduced by Dunn *et al.* is one of the most famous systems and is already used in clinical practices (3). Bioabsorbable sucrose acetate isobutyrate (SAIB) is also used as the non-polymeric matrix ISFIs systems. SAIB is a mixture of the reaction products formed by the esterification

Electronic supplementary material The online version of this article (doi:10.1208/s12249-015-0292-2) contains supplementary material, which is available to authorized users.

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of food-grade sucrose with acetic anhydride and isobutyric anhydride (see Fig. 1). SAIB is a water-insoluble, viscous liquid with a viscosity of approximately 100,000 mPa s, which has been approved as a food additive to stabilize emulsions. SAIB can be diluted with small amounts (15–35%) of pharmaceutically acceptable organic solvents, such as ethanol, dimethyl sulfoxide (DMSO), or *N*-methyl-2-pyrrolidone (NMP), to form low-viscosity solutions (50 to 200 mPa s), which can be readily injected via small-gauge needles. ISFIs based on SAIB incorporated with different active ingredients, including bupivacaine, human growth hormone, risperidone, etc., were tested for its applicable as injectable depots (13,14). SABER™-Bupivacaine (POSIDUR™) was developed by Southern Biosystems for local treatment of post-surgical pain. POSIDUR™ is used to continuously release bupivacaine and provide up to 3 days of uninterrupted local anesthesia, which has been in clinical phase III trials (15).

One of the challenges in translating ISFIs systems from bench to clinic is to control the initial burst release. Several formulation parameters, such as the types, molecular weight, and concentrations of the matrix materials, amount of organic solvent incorporated, should be optimized to minimize the burst effect (16).

In this paper, two different types of ISFIs containing cucurbitacin (Cuc) as the model drug were prepared using PLGA and SAIB as the matrix materials (PLGA ISFIs and SAIB ISFIs), and their physiochemical properties, *in vivo* pharmacodynamics and pharmacokinetics after intratumoral administration to melanoma bearing mice were investigated. Cuc is a type of triterpenoid compound isolated from members of the *Cucurbitaceae* family of plants that have been used as anti-inflammatory and anti-diabetic agents in China, and Cuc-B is the main component of Cuc (about 60% in the commercial raw Cuc). Many studies proved that Cuc could inhibit the growth of a wide spectrum of human malignant cells both *in vitro* and in xenografted tumor models (17). Compared to the drug-loaded PLGA particle systems, the ISFIs were expected to be able to reside in the injection site in tumor (minimum leakage) and favorable drug release profiles, and subsequently better antitumor efficiency.

MATERIALS AND METHODS

Materials

SAIB (density of 1.146 g/ml at 25°C) was purchased from Sigma Aldrich (St. Louis, MO, USA). PLGA 50/50 (inherent viscosity=0.50 dl/g in $CHCl_3$ at 25°C) was a kind gift from Changchun SinoBiomaterials Co., Ltd (Changchun, China). Cucurbitacin (Cuc, Cuc-B content: 61.5%) was purchased from Tianjin Institute of Pharmaceutical Research (Tianjin, China). DMSO and ethanol were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). NMP (Pharmasolve®) was a gift from ISP Technologies, Inc. (NJ, USA). All other chemicals and solvents were of analytical or chromatographic grade.

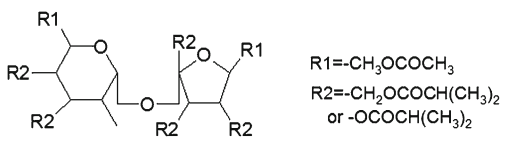


Fig. 1. The chemical structure of SAIB

The B16 murine melanoma cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and the A375.S2 human melanoma cell line was a kind gift from Professor Takshi Ikejima at Shenyang Pharmaceutical University. Two cell lines were cultured in RPMI 1640 supplemented with 10% FBS. C57BL/6 male mice and BALB/c-nu male mice were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University (Liaoning, China), and used at 6~8 weeks age. All animal experiments were conducted in accordance with the ethical guidelines on animal experiments of Shenyang Pharmaceutical University.

Preparation of ISFIs

PLGA ISFIs and SAIB ISFIs were prepared with similar methods as described in literatures with appropriate modifications (13,18,19). Briefly, PLGA or SAIB was dissolved in certain volume of organic solvent, Cuc powder was then added under constant stirring until the formation of homogeneous solution. The formulations of ISFIs prepared are listed in Table I.

In Vitro Drug Release

Accurately weighed cuc-loaded ISFIs (about 0.1 g) were placed into 5-ml test tubes, and 4 ml release medium was added (50 mM PBS pH 6.8 with 0.02% NaN_3). The tubes were then incubated in a reciprocating water bath shaking incubator (ZHWY 110X30, Zhicheng Inc., Shanghai, China) at 100 rpm and 37°C (13,20). Three milliliters of samples was withdrawn at predetermined time intervals and replaced with fresh release medium. The drug concentration (calculated as Cuc-B) was analyzed by HPLC (Shimadzu LC-10A, Kyoto, Japan) as previously described (1). All experiments were performed in triplicate.

Diffusion of Organic Solvent

The diffusion kinetics of solvent into the release medium was investigated by determining the refractive index of the medium at predetermined time intervals (20,21). The procedure and devices used were the same as in the drug release experiments. At certain time intervals, 100 μl of sample was withdrawn and replaced with the same amount of fresh medium. The refractive index of the sample was determined using a 2WAJ Abbe Refractometer (Jingke, Shanghai China). The amount of solvent diffused into the release media was calculated from a calibration curve over the range of 1 to 30 mg/ml. All experiments were performed in triplicate.

Rheological Evaluation

The dynamic viscosity of ISFIs was strongly associated with the organic solvent type and may greatly affect the initial burst release. The rheological properties of various ISFIs were tested by an AR2000ex Rheometer (TA Instruments, USA) using parallel-plate mode at 25°C. The shear rate was programmed with a linear shear rate increasing from 0 to 100 s^{-1}

Table I. Formulations of PLGA ISFIs and SAIB ISFIs

	ISFIs	Solvents	Matrix content (%, *w*/*w*)	Solvent content (%, *w*/*w*)	Cuc content (mg/g)
SAIB ISFIs	SAIB-1	Ethanol	85.0	15.0	2.0
	SAIB-2	NMP	85.0	15.0	2.0
	SAIB-3	DMSO	85.0	15.0	2.0
	SAIB-4	NMP	50.0	50.0	2.0
	SAIB-5	NMP	85.0	15.0	0.5
	SAIB-6	NMP	85.0	15.0	6.0
PLGA ISFIs	PLGA-1	DMSO	33.0	67.0	2.0
	PLGA-2	NMP	33.0	67.0	2.0
	PLGA-3	NMP	25.0	75.0	2.0
	PLGA-4	NMP	16.7	83.3	2.0
	PLGA-5	NMP	33.0	67.0	0.5
	PLGA-6	NMP	33.0	67.0	6.0

within 1 min, and after keeping a constant shear rate of 100 s^{-1} for 10 s, the shear rate was then decreased linearly to 0 s^{-1} within 1 min. The response of stress and dynamic viscosity to shear rate were recorded and analyzed with the software.

Sol–Gel Transition of ISFIs

Real-time optical microscopic observation was performed to investigate the sol–gel transition of Cuc-loaded ISFIs. Experiments were carried out to mimic the *in vivo* transition process. Briefly, one drop of SAIB ISFIs or PLGA ISFIs was placed between the glass slide and cover glass with a gap of about 1 mm, as shown in Fig. 2. The device was then immersed in the release medium containing 10 μg/ml crystal violet to enhance the contrast. The sol–gel transition process was recorded by light microscopy at room temperature (Motic® Optical BA 300 Pol., Motic Inc., China).

SEM Observation for Solidified PLGA ISFIs

The influence of solvents (NMP and DMSO) on the morphologies of the solidified implants of PLGA ISFIs (PLGA:solvent=1:2) was investigated by SEM observation. After drug release, the *in situ* formed implant samples were freeze-dried and sputter-coated with gold, and then observed by scanning electron microscopy (Hitachi S-3400, Hitachi High Technologies, Kyoto, Japan).

Pharmacokinetics in Tumor-Bearing Mice

The drug levels (calculated as Cuc-B) in tumor and in plasma after intratumoral administration of ISFIs were measured as described previously (1). Briefly, Cuc-loaded SAIB ISFIs and PLGA ISFIs were intratumorally injected (single-point injection, equivalent to 0.2 mg Cuc/mouse). For comparison purpose, the pharmacokinetics of multi-points injection (three points injection in tumor, equivalent to 0.2 mg Cuc/mouse denoted as SAIB ISFIs-se) of SAIB ISFI was investigated. The area under the curve ($AUC_{0-28\ days}$) was calculated by the trapezoidal rule from zero to the last sampling time.

Pharmacodynamic Studies

The pharmacodynamics after single-point intratumoral injection of SAIB ISFIs and PLGA ISFIs, and three-point injection of SAIB ISFIs was evaluated in B16 cell-inoculated C57BL/6 mice (ten mice for each group). Briefly, 1×10^5 B16 cells were subcutaneously injected into the right flank of the C57BL/6 mice. Treatments began on the day when the tumor volume was over 200 mm^3. Seven groups were defined as follows: (I) Control-saline group, treated with intraperitoneal injections of normal saline; (II) Control-NMP group, treated with blank SAIB ISFIs using NMP as the solvent; (III) positive control group, treated with intraperitoneal injection of cyclophosphamide (0.2 mg/mouse/day); (IV) Cuc group, treated with intratumoral injection of raw Cuc (0.2 mg/mouse); (V) PLGA ISFIs group, treated with intratumoral injection of

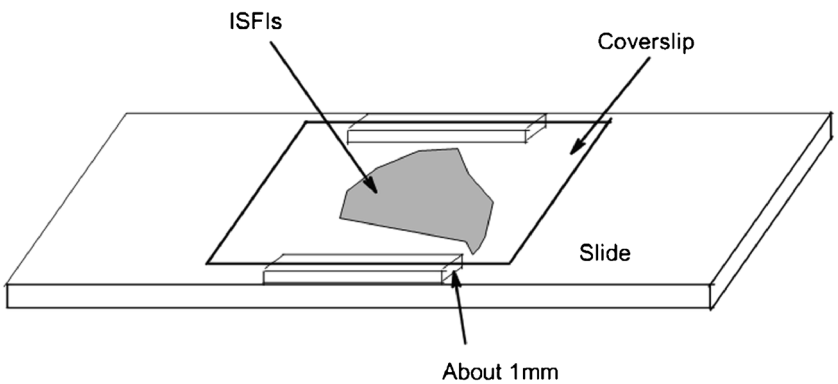


Fig. 2. Schematic diagram of the device for the real-time observation of the sol–gel transition of ISFIs with optical microscope

Cuc-loaded PLGA ISFIs (equivalent to 0.2 mg Cuc/mouse); (VI) SAIB ISFIs group, treated with intratumoral injection of Cuc-loaded SAIB ISFIs (equivalent to 0.2 mg Cuc/mouse); and (VII) SAIB ISFIs-se groups, treated with three-point intratumoral injection of Cuc-loaded SAIB ISFIs (equivalent to 0.2 mg Cuc/mouse). The tumor-bearing mice were euthanized 14 days after the first injection, and the tumor was dissected and weighed to calculate the tumor inhibition ratio.

For further evaluation of the anti-melanoma efficiency of SAIB ISFIs, human malignant melanoma cell A375.S2 xenografted nude mice model was used. Male BALB/c-nu mice were selected in the present study. The tumor inoculation and treatment procedures were the same as for the C57BL/6 mice model. Four groups were defined as follows: (I) control group, treated with intraperitoneal injection of normal saline; (II) positive control group, treated with intraperitoneal injections of cyclophosphamide (0.2 mg/mouse/day); (III) SAIB ISFIs groups, treated with intratumoral injection of Cuc-loaded SAIB ISFIs (equivalent to 0.2 mg Cuc/mouse); and (IV) SAIB ISFIs-se group, treated with three-point intratumoral injection of Cuc-loaded SAIB ISFIs (equivalent to 0.2 mg Cuc/mouse).

The tumor size was measured at predetermined time points, the tumor volume was determined with Eq. 1:

$$\text{Tumor volume}\left(\text{mm}^3\right) = \left[A(\text{mm}) \times B^2\left(\text{mm}^2\right)\right]/2 \quad (1)$$

where A is the largest dimension of the tumor, and B is the smallest dimension. The tumor volume inhibition ratio was calculated with Eq. 2:

$$\left(V_{\text{time point}}/V_{\text{intial}}-1\right) \times 100\% \quad (2)$$

The tumor volume doubling time (DT) was calculated with Eq. 3 (22):

$$\text{DT(day)} = T \times \log 2/(\log V_F - \log V_I) \quad (3)$$

where V_F is the final tumor volume, V_I is the initial tumor volume, and T is the time difference between the initial and final day.

Pilot Studies on Animal Survival

The effects of intratumoral Cuc-loaded ISFIs on overall animal survival were investigated in tumor-bearing C57BL/6 mice. The treatments were the same as those in the pharmacodynamic studies. The percent increase in median life span (ILS) was calculated with Eq. 4:

$$\text{ILS} = \left(\text{Mean survival time}_{\text{treat group}}/\text{Mean survival time}_{\text{control group}}-1\right) \times 100\% \quad (4)$$

Statistical Analysis

Data are shown as mean±SD. Data were checked for outliers and the normality of data distribution. The analysis of therapeutic effects, including the tumor volume inhibition ratio, final tumor mass, and the results of quantitative analysis were performed using one-way ANOVA with Tukey's post hoc test for pair-wise comparison. Significant differences between survival rates of the animals were determined by Kaplan–Meier analysis. Values of $p<0.05$ were considered significant. Statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL) (23).

RESULTS AND DISCUSSION

Preparation and *In Vitro* Drug Release

The PLGA ISFIs and SAIB ISFIs obtained were colorless and syrup-like solutions containing readily dissolved Cuc.

After injection into the local body, the hydrophilic organic solvent began to diffuse out from the ISFIs, the hydrophobic matrix precipitated and trapped the drug in the formed implant (7). The release kinetics of organic solvent from the ISFIs may strongly affect the initial drug release (before the implants completely solidified).

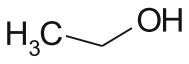
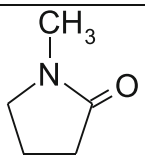
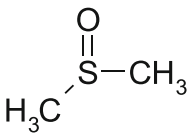
The most commonly used solvents in ISFIs include ethanol, NMP, and DMSO. Biocompatibility studies of PLGA solutions in NMP and DMSO in rhesus monkeys showed no acute toxicity or tissue reaction (16). According to our previous report (13), ethanol, NMP, and DMSO were the suitable solvents for SAIB ISFIs. And based on the manufacturer's information for Atrigel® system and other references (1–5), NMP and DMSO were selected for the solvent of PLGA ISFIs system in this study. The basic properties of these solvents are listed in Table II. Of these three solvents, NMP has the highest boiling temperature and lower LD_{50} value.

The effects of solvent on drug release of SAIB ISFIs and PLGA ISFIs are presented in Fig. 3a, b, respectively. For SAIB ISFIs, the order of the initial release percents (the first day) was as follows: ethanol (17.34±0.99%)>NMP (11.12±1.33%)≈DMSO (10.36±1.00%). And the accumulated drug release percents (28 days) of ISFIs containing ethanol, NMP, and DMSO were 50.61±1.08%, 50.38±2.77%, and 54.42±2.88%, respectively, which showed no significant differences between SAIB ISFIs with different solvents. For PLGA ISFIs using NMP and DMSO as the solvents, the initial release percents were 32.44±2.17% and 34.71±1.16%, and the accumulated release percents at 28 days were 57.45±1.37% and 43.82±1.48%, respectively. Due to the poor solubility of PLGA in ethanol, no results were obtained for ISFIs of PLGA-ethanol system.

For ISFIs, factors such as the amount of drug loaded, the types of matrix materials, and the effective area for drug release would affect the release patterns of the ISFIs. As summarized in literatures, three mechanisms/steps occur in releasing sequence of PLGA ISFIs system: (1) burst release, (2) relaxation-induced drug dissolution controlled release, and (3) diffusion release. At any time, the step with the lowest rate becomes the rate limiting step and ultimately controls the overall drug release rate (25–29).

In our study, for SAIB ISFIs with different drug loading, the percentage of drug released showed no significant difference, while for PLGA ISFIs the percentage of drug released decreased with the increase of drug loading (Fig. 3c). This result may be attributed to the different release patterns due

Table II. The Properties of Solvents Used for Various ISFIs (24)

	Ethanol (absolute)	NMP	DMSO
Structure			
Boiling Temp. (°C)	78.5	202	189
Dynamic viscosity (mPa s)	1.22 (20°C)	1.65 (25°C)	2.47 (20°C)
LD_{50} (mouse, ip., g/kg)	0.93	3.05	2.5

to the different solidification rates of these two ISFI systems as discussed in "Sol–Gel Transition of ISFIs". Many studies have reported that the solvent–matrix ratio affected the release patterns of ISFIs, and reported that *in vitro* drug release from ISFIs system showed marked difference between the miscible solvents and the partially miscible solvents, and the amount of drug released decreased with the increase of polymer concentration (27,29–32). The effect of the solvent–matrix ratios on the release pattern of the tested ISFIs are showed in Fig. 3d, e. The amount of drug released at the initial stage increased with the increase of solvent in the ISFIs.

Furthermore, the effects of the surface area of ISFIs on drug release were illustrated in Fig. 3f (ISFI-se, i.e., implants formed by three-point injection for *in vitro* release, presented great larger surface area compared to that of single-point injection). The drug release rate of PLGA ISFI-se was obviously lower than that of PLGA ISFI, and an interesting finding is that SAIB ISFI and SAIB ISFI-se showed very similar drug release patterns. This result may be attributed to the rapid solvent diffusion of PLGA ISFI-se, consequently, the rapidly solidified PLGA matrix hindered drug release. The solidification mechanism for the SAIB ISFIs was different to that of PLGA ISFIs, which will be discussed in detail below.

Comparing to PLGA ISFIs, the initial burst release of SAIB ISFIs was relatively slower (see Fig. 3g). This result may partly be attributed to the different molecular structures of Cuc, PLGA, and SAIB, which were triterpenes, linear aliphatic polyester, and ester of sucrose, respectively. The strong stereo resistance of the sterical cyclohexanal skeletal rings in both Cuc and SAIB molecules may be the main reason for the lower initial burst release of SAIB ISFIs.

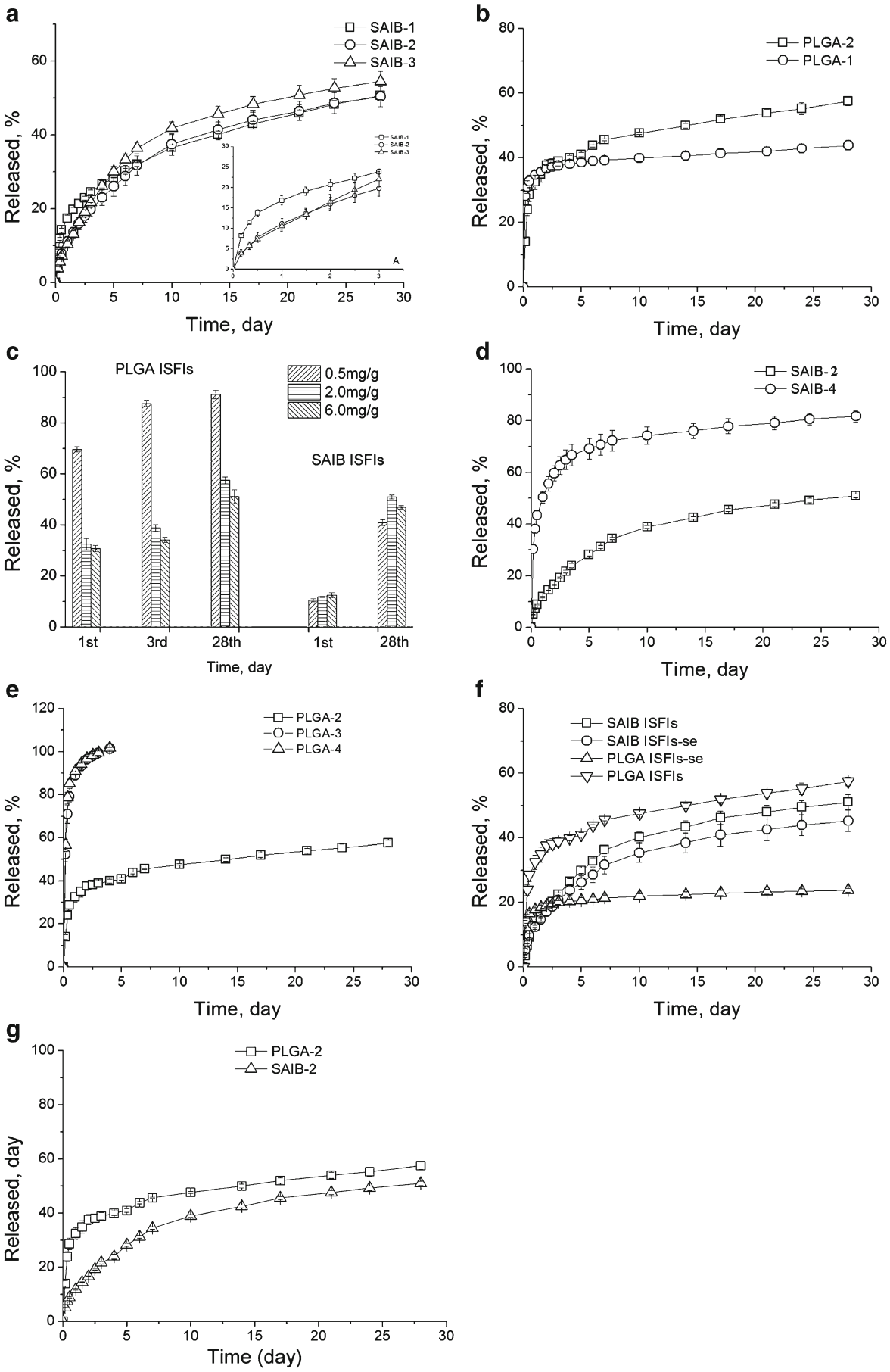
Diffusion of Organic Solvent

The solvent diffusion dynamics displayed an important role in the formation of ISFIs, as well as to the initial drug release (30–32). Meanwhile, it is believed that the burst release of ISFIs was strongly affected by the matrix gelation kinetics. The gelation kinetic parameters including the water influx rate (the diffusion of water from the physiologic surroundings and subsequent accumulation within the injected ISFIs), the organic exflux rate (the organic solvent diffused out from the matrix) and the matrix gelation rate (the rate at which the solution is transformed into a solidified implant) (30,31). The diffusion kinetics of organic solvent (see Fig. 4) from ISFIs into the release medium was investigated in order to obtain further insight into the underlying drug release mechanisms.

The differences on the initial solvent diffusion may be attributed to the solvent–matrix interaction and different solvent intrinsic viscosity (29,32). For SAIB ISFIs, the diffusion of ethanol from the ISFIs was much higher than NMP and DMSO; 85.54±5.51% of ethanol was released within the first hour, compared to 29.35±4.06% of NMP and 24.70±1.23% of DMSO, within the first hour. And 100% of ethanol was released after 12 h. The diffusion of NMP and DMSO showed no significant differences. The different initial solvent diffusion for SAIB ISFIs may due to the different intrinsic viscosities of the solvents. For PLGA ISFIs system, the diffusion of DMSO was slower than that of NMP. Compared to DMSO, about more than 15% of NMP was diffused in the first hour, while the solvents diffused out almost completely after 24 h. Meanwhile, no differences on the initial drug release stage were observed in all the PLGA systems investigated (Fig. 3c). For these two types of ISFIs systems, the diffusion of organic solvents in SAIB system was slower than PLGA system, which may due to the larger amount of solvent presented in the PLGA systems or the different diffusion pattern as described in the section "Sol–Gel Transition of ISFIs".

Rheological Properties of SAIB ISFIs and PLGA ISFIs

As reported in literatures (13,20), the solvent diffusion rate is affected by the viscosity of the ISFIs during phase change. The rheological property of ISFIs is an important factor that influences the solidification and initial drug release of ISFIs (33,34). Figure 5a illustrated that the shear stress of SAIB ISFIs increased with the increase of the shear rate in a linear fashion. SAIB solutions in ethanol, DMSO and NMP can therefore be defined as Newtonian fluids, and the dynamic viscosities of the SAIB ISFIs with different solvents follow the order: DMSO>NMP>ethanol. The lower viscosity, which indicates relatively weak intermolecular interaction between solvent and matrix, and meanwhile related to the intrinsic viscosity of the solvents (see Table II), can explain the higher burst release of the SAIB–ethanol system (35). The PLGA ISFIs using both NMP and DMSO presented the rheological behavior of gel-like fluids, and the system with DMSO showed


a
SAIB-1
SAIB-2
SAIB-3
Released, %
Time, day
b
PLGA-2
PLGA-1
Released, %
Time, day
c
PLGA ISFIs
0.5mg/g
2.0mg/g
6.0mg/g
SAIB ISFIs
Released, %
1st
3rd
28th
1st
28th
Time, day
d
SAIB-2
SAIB-4
Released, %
Time, day
e
PLGA-2
PLGA-3
PLGA-4
Released, %
Time, day
f
SAIB ISFIs
SAIB ISFIs-se
PLGA ISFIs-se
PLGA ISFIs
Released, %
Time, day
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PLGA-2
SAIB-2
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◀ **Fig. 3.** The *in vitro* Cuc release profiles of ISFIs. **a** SAIB ISFIs with different organic solvents; **b** PLGA ISFIs with different organic solvents; **c** effects of drug loaded on release, **d** SAIB ISFIs with different matrix-solvent ratio, **e** PLGA ISFIs with different matrix-solvent ratio, **f** SAIB ISFIs and PLGA ISFIs released with different contacted surface (formulation as SAIB-2 and PLGA-2), and **g** comparison of PLGA ISFIs and SAIB ISFIs

higher viscosity. The shearing thinning appeared at lower shear rate region, which means that higher force will be required to squeeze out the PLGA ISFIs from the syringes.

Due to the lower toxicity of NMP, and the appropriate physiochemical properties and drug release patterns of the ISFIs using NMP as the solvent as described above, for further pharmacokinetic and pharmacodynamic studies, NMP was used to generate the ISFIs.

Sol–Gel Transition of ISFIs

Under optical microscope, different morphological changes were observed for SAIB ISFIs and PLGA IFSIs, which were related to the different initial drug release for these two types of ISFIs.

With the influx of water, water-rich zones inside ISFIs and gelation layer can be observed (Fig. 6) in SAIB ISFIs, and then the water-rich zone would migrate outward. The gelation outer shell formed thus prevents the water-rich zone from migrating, which results a reduction of the initial burst. The estimated thickness of the gelation shells of SAIB ISFIs with different solvents were 30.2 μm (ethanol, Fig. 6a), 10.7 μm (NMP, Fig. 6b), and 11.1 μm (DMSO, Fig. 6c). The tenacious and unclear gelation shell of SAIB–ethanol system was due to the lower viscosity and higher miscibility of ethanol with water.

While for PLGA IFSIs, the solidification of the systems took place on the order of seconds to minutes. Fast-phase transition of the polymer solutions and high affinity of the formed depot to solvent, combined with the solvent diffusion forced by the concentration gradient, resulted to finger-like channels inside the implants (Fig. 6d–f). The diameter of the channels in the PLGA–NMP system was larger than that of the PLGA–DMSO system. Furthermore, higher solvent content resulted to larger diffusion channels.

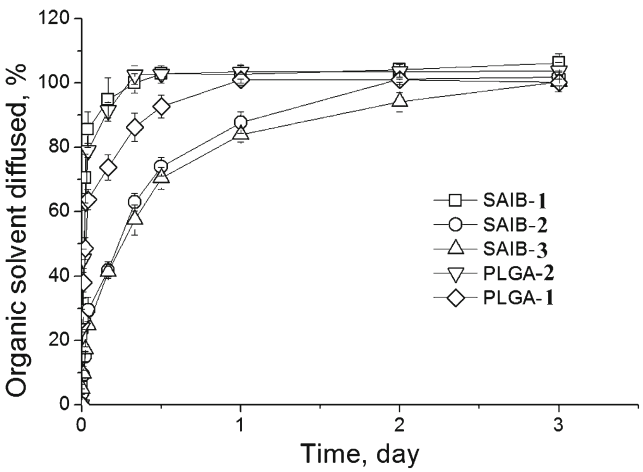

Fig. 4. The diffusion of the organic solvents from various ISFIs into the release medium

Morphologies of PLGA ISFIs after Drug Release

Gao (36) reported that the diffusion channels around the matrix formed after contact with the aqueous environment might modulate the initial burst release of drug. The surface and cross-section morphologies of the PLGA ISFIs were observed as presented in Fig. 7. Compared to the PLGA ISFI with DMSO, the channels of PLGA ISFI with NMP was larger and more regularly aligned with the open end to the surface, which may reduce the tortuous diffusion pathway of drug molecules. The morphologies of the cross-section of these two systems showed no apparent differences.

Pharmacokinetics of ISFIs in B16 Inoculated C57BL/6 Mice

The drug concentrations in tumor and in plasma as functions of time after intratumoral injection of cuc-loaded SAIB ISFIs, PLGA ISFIs, and SAIB ISFIs-se into B16 tumor-bearing mice are shown in Fig. 8a, b. The C_{max} values in tumor after administration of SAIB ISFIs, SAIB ISFIs-se, and PLGA ISFIs were 17.23±1.33 μg/g, 17.28±1.86 μg/g, and 24.79±3.73 μg/g, respectively, which were all much lower than that of intratumoral injection of raw Cuc (42.64±2.98 μg/g).

The AUC value represented the drug amount exposure over certain time duration. In this study, the drug exposed in tumor (represented as $AUC_{tumor,\ 0–28\ days}$) and in blood (represented as $AUC_{plasma,\ 0–28\ days}$), and the ratio of AUC_{plasma} to AUC_{tumor} ($AUC_{plasma,\ 0–28\ days}$ /$AUC_{tumor,\ 0–28\ days}$) are listed in Table III. The AUC_{plasma}/AUC_{tumor} ratios may be used to estimate the benefits-risk ratio after the intratumoral injections. The lower value of the AUC_{plasma}/AUC_{tumor} ratio indicates the higher amount of drug in tumor to display the therapeutic effects, thus smaller amount of drug in systemic circulation to induce side effects. As a viscous and adhesive material of SAIB (see the Supplementary Fig. 1), the lower leakage from the pinhole and $AUC_{plasma,\ 0–28\ days}/AUC_{tumor,\ 0–28\ days}$ ratio (and also the best antitumor efficiency in pharmacodynamic studies) can be expected after intratumoral injection of SAIB ISFIs. The higher $AUC_{plasma,\ 0–28\ days}/AUC_{tumor,\ 0–28\ days}$ ratio of PLGA ISFIs can be attributed to the high initial burst rather than the lower solidification rate in tumor. Figure 8c showed the residual amount of drug in ISFIs after intratumoral administration of SAIB ISFI, SAIB ISFIs-se, and PLGA ISFI, and the percentage of drug in tumor and percentage of drug released indicated correlations with the linear fitting indexes of more than 0.9 (Fig. 8d).

Pharmacodynamics of ISFIs in Tumor-Inoculated Mice

The pharmacodynamics after intratumoral injection of Cuc-loaded ISFIs to B16-bearing mice was evaluated by indices including body weight growth ratio, tumor volume increase ratio, tumor volume doubling time, and mouse survival time (Fig. 9). The body weight growth ratios of SAIB ISFIs-se group were compared to controls and PLGA ISFIs groups, which showed significant differences ($p<0.05$) at the 28th day (Fig. 9a). Figure 9b showed the tumor volume growth as a function of time after single dose treatment of ISFIs. SAIB ISFIs-se displayed significant inhibitory effects against B16 tumor compared with control groups (both saline and

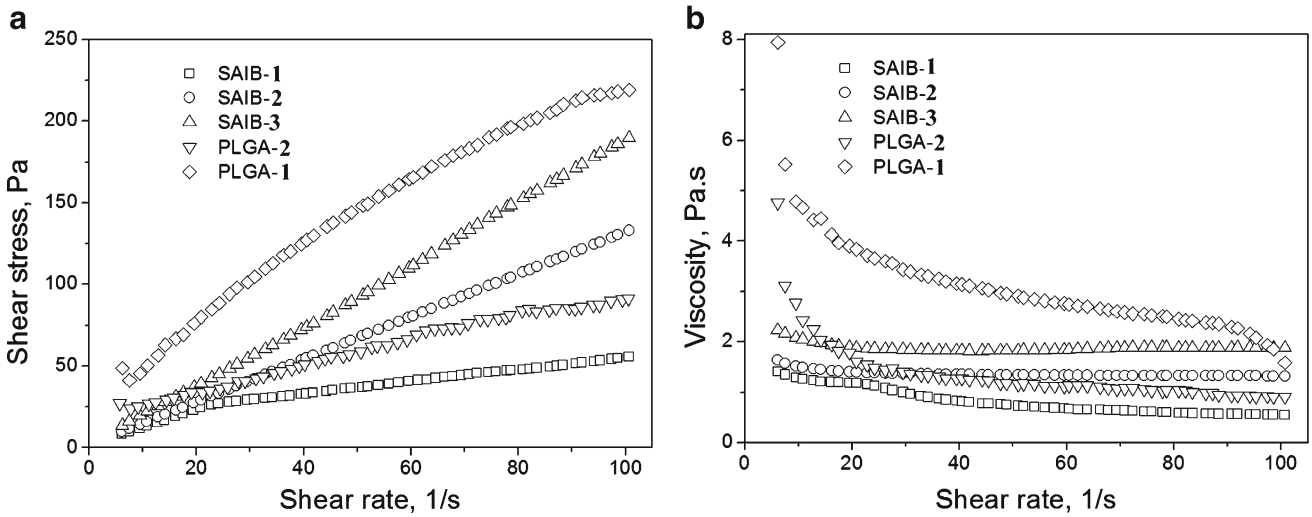

Fig. 5. The rheological properties of ISFIs. **a** Plots of shear stress to shear rate, and **b** plots of viscosity to shear rate

NMP groups) and PLGA ISFIs group ($p<0.05$). The tumor volume doubling time of different treated groups was shown in Fig. 9c, and the SAIB ISFIs-se group displayed the longest doubling time compared with control groups and other treated groups ($p<0.05$). The survival increase ratio suggests that SAIB ISFIs-se is more efficacious than other groups in inhibiting melanoma growth (Fig. 9d).

The pharmacodynamic studies were performed on human melanoma cell A375.S2 xenografted mice treated with SAIB ISFIs and SAIB ISFIs-se. The body weight growth ratio, tumor volume

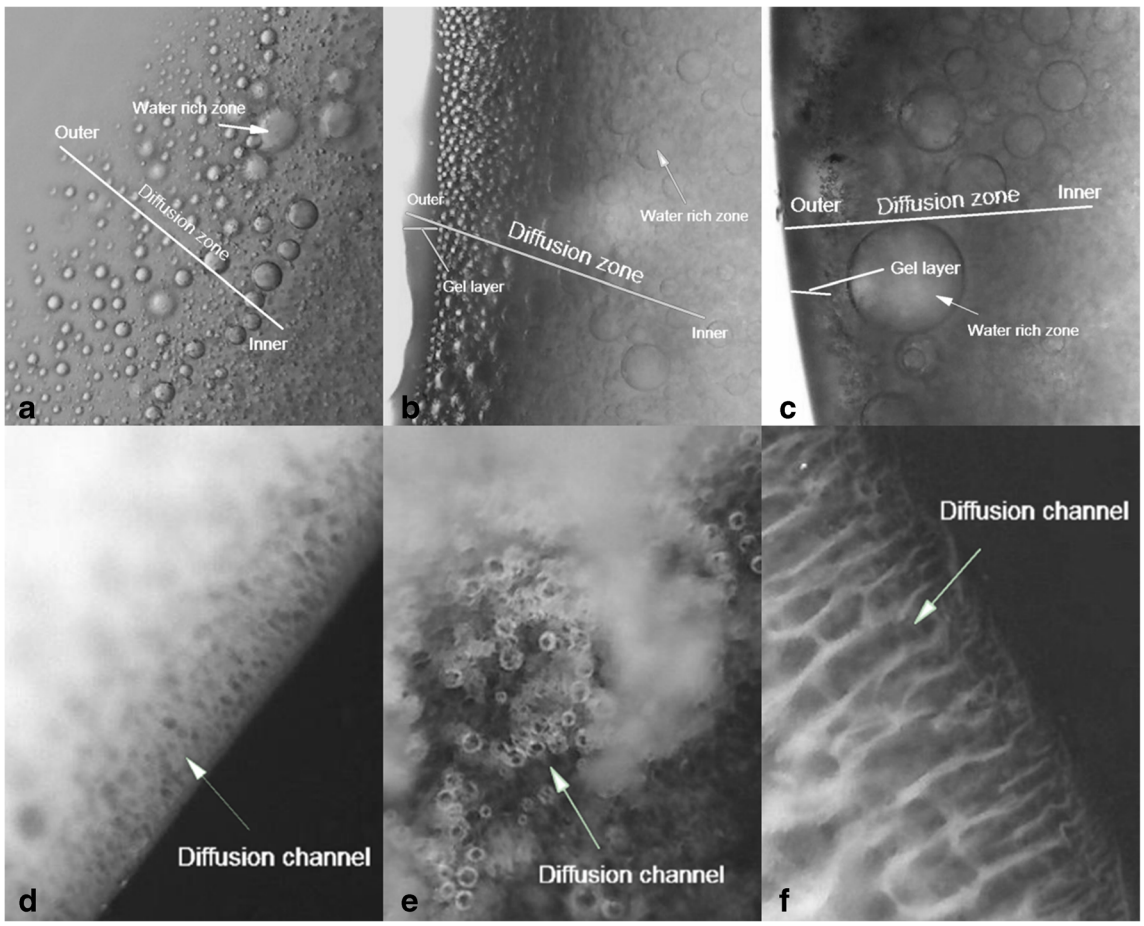

Fig. 6. The optical microscopic images of ISFIs contact to the release media. **a** SAIB-1, **b** SAIB-2, **c** SAIB-3, **d** PLGA-1, **e** PLGA-2, and **f** PLGA-4 (for formulations, see Table I. The magnifications were ×400 and ×100 for SAIB ISFIs and PLGA ISFIs, respectively. The videos provided in the supplementary material displayed the real-time observations on the exactly sol–gel transition processes of ISFIs.)

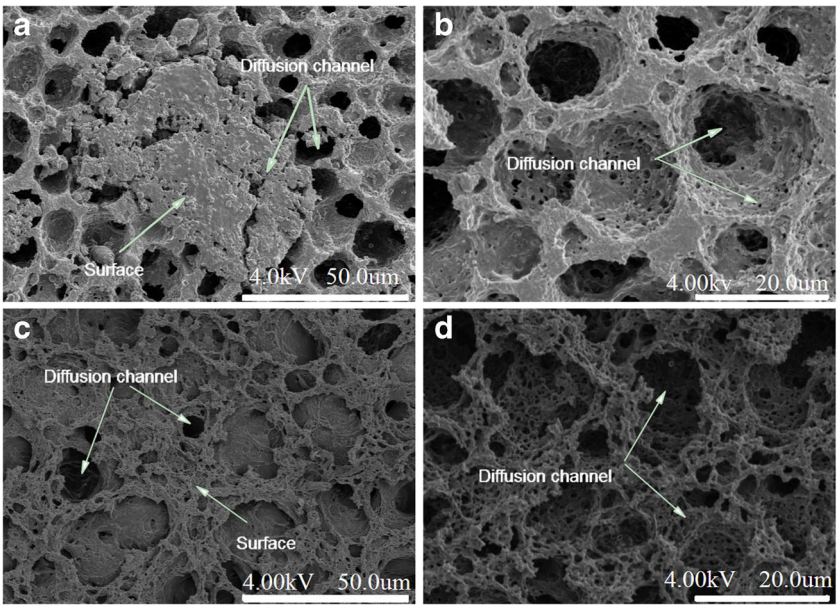

Fig. 7. The SEM images of PLGA ISFIs after drug released for 28 days. **a** Surface, PLGA ISFIs with NMP, **b** Cross-section, PLGA ISFIs with NMP, **c** Surface, PLGA ISFIs with DMSO, and **d** Cross-section, PLGA ISFIs with DMSO

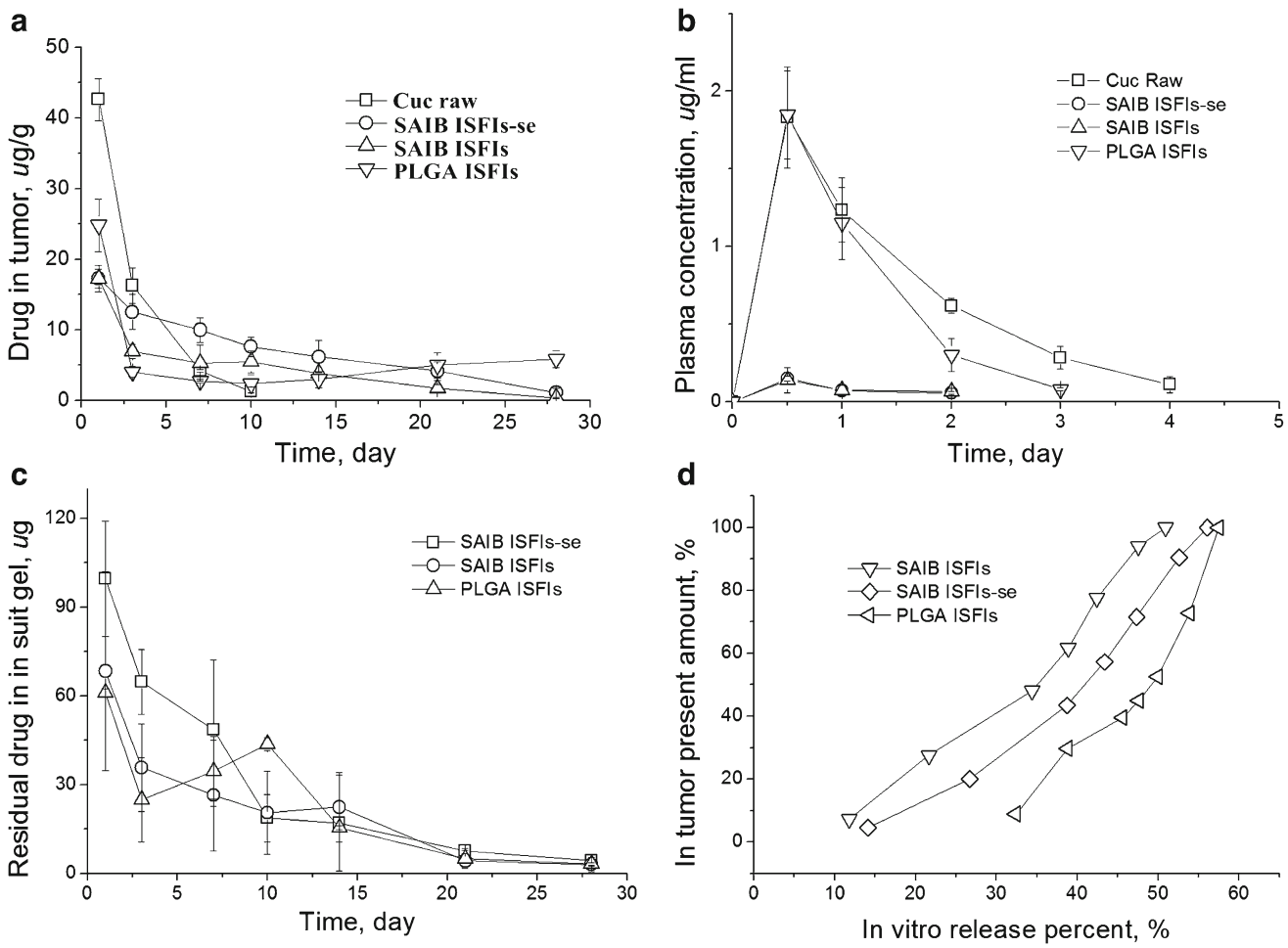

Fig. 8. The pharmacokinetics of Cuc and Cuc-loaded ISFIs after intratumoral injection to B16 bearing mice. **a** The drug concentrations in tumor versus time *curves*, **b** The drug concentration in plasma versus time curves, **c** The residual drug in ISFIs versus time *curves*, and **d** The relationships between the percentage of drug in tumor and percentage of drug released

Table III. Parameters of *In Vitro* Release and *In Vivo* of Cuc (calculated as Cuc-B) After Intratumoral Injection of Various ISFIs

	Cuc	SAIB ISFIs	SAIB ISFIs-se	PLGA ISFIs
Burst released (%, 3 days)	100%	19.78±1.94	20.76±1.30	38.84±1.33
Total released (%, 28 days)	–	50.38±2.77	45.27±3.25	57.45±1.37
$AUC_{tumor,\ 0–28\ days}$ (μg·day/g)	129.22	118.87	191.93	138.63
$AUC_{plasma,\ 0–28\ days}$ (μg·day/ml)	2.80	0.16	0.16	2.13
$AUC_{plasma,\ 0–28\ days}/AUC_{tumor,\ 0–28\ days}$ (%)	2.17	0.13	0.08	1.54

increased ratio, tumor doubling time, and finally tumor mass were selected to evaluate the inhibition efficiency of different treatment groups (Fig. 10). The body weight loss (Fig. 10a) was due to the malignant melanoma tumor injuries caused by A375.S2 cells. SAIB ISFIs-se group displayed the best efficiency in inhibiting weight loss compared with other treatment groups ($p<0.01$, compared to control group and positive group). Compared to other treatment groups, SAIB ISFIs-se also displayed the best inhibition efficiency in tumor growth as measured by tumor volume increase ratio and tumor mass ($p<0.05$). And only SAIB ISFIs-se group displayed significant differences in tumor volume doubling time compared to the control group in our experiments. Commonly, it had been reported that the tumor inhibition in the pharmacodynamic studies is greatly dependent on the tumor shape (37). For example, for the radiotherapy, the volume of spherical-shaped tumor decreased significantly compared to the irregular shaped tumor. However, multi-points intratumoral injection seemed to be able to overcome this obstacle; in our present study, multi-points intratumoral injection (three points) of SAIB ISFIs displayed the best anticancer efficiency.

Kluza (38) reported that the initial tumor volume strongly influenced the final tumor volume and supported a positive correlation between the pre- and post-treatment tumor volume, which could partly explain the unsatisfactory results of the tumor growth inhibition of our study in xenograft models. In our experiments, the treatment was set to begin after the tumor volume was larger than 200 mm^3 in order to ease the intratumoral injection, while the initial tumor volume was normally less than 100 mm^3 in most studies.

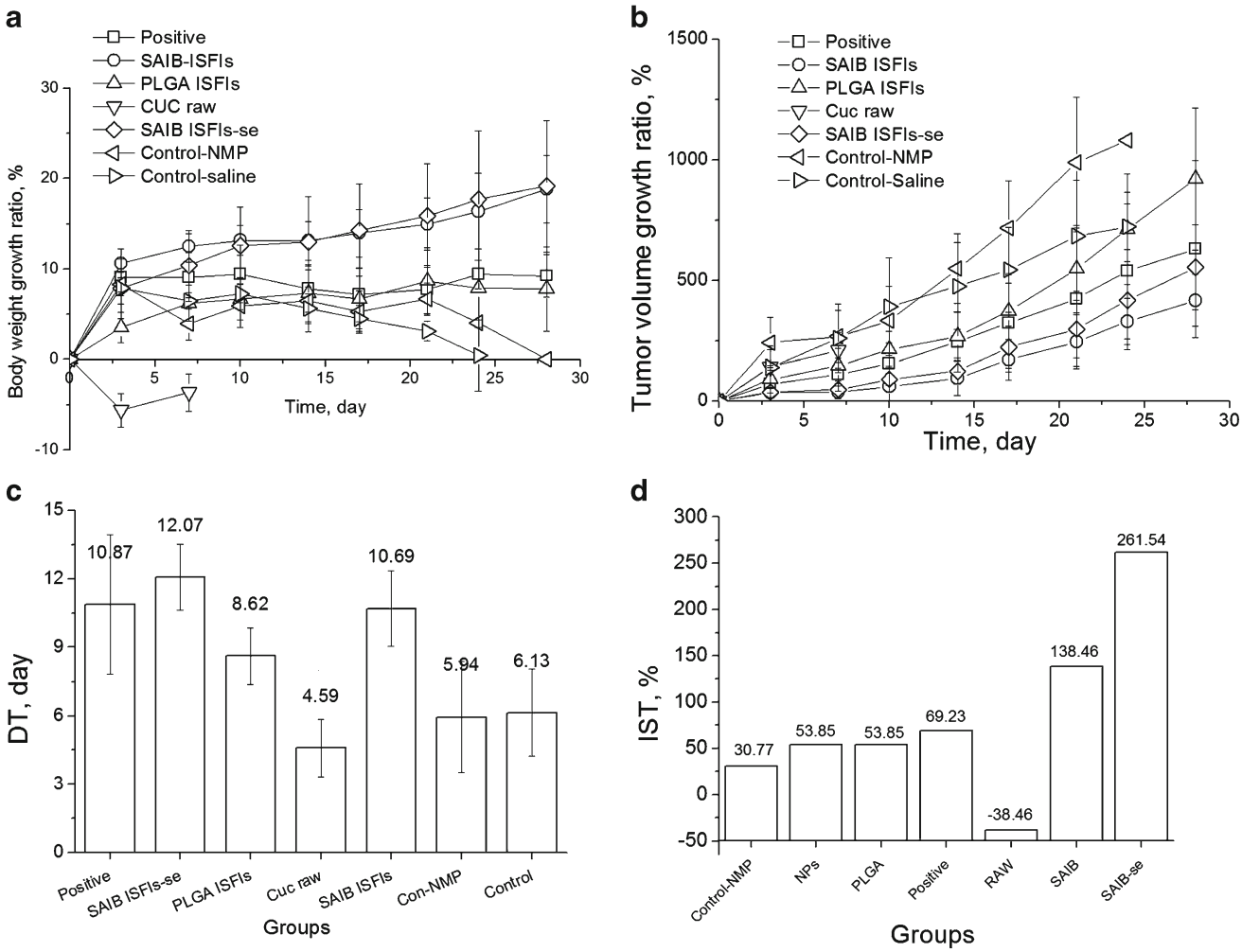

Fig. 9. The anti-melanoma efficiencies of Cuc and Cuc-loaded ISFIs after intratumoral injection to B16 cell bearing mouse. **a** Body weight growth ratio, **b** tumor volume increased ratio, **c** tumor volume doubling time, and **d** IST values

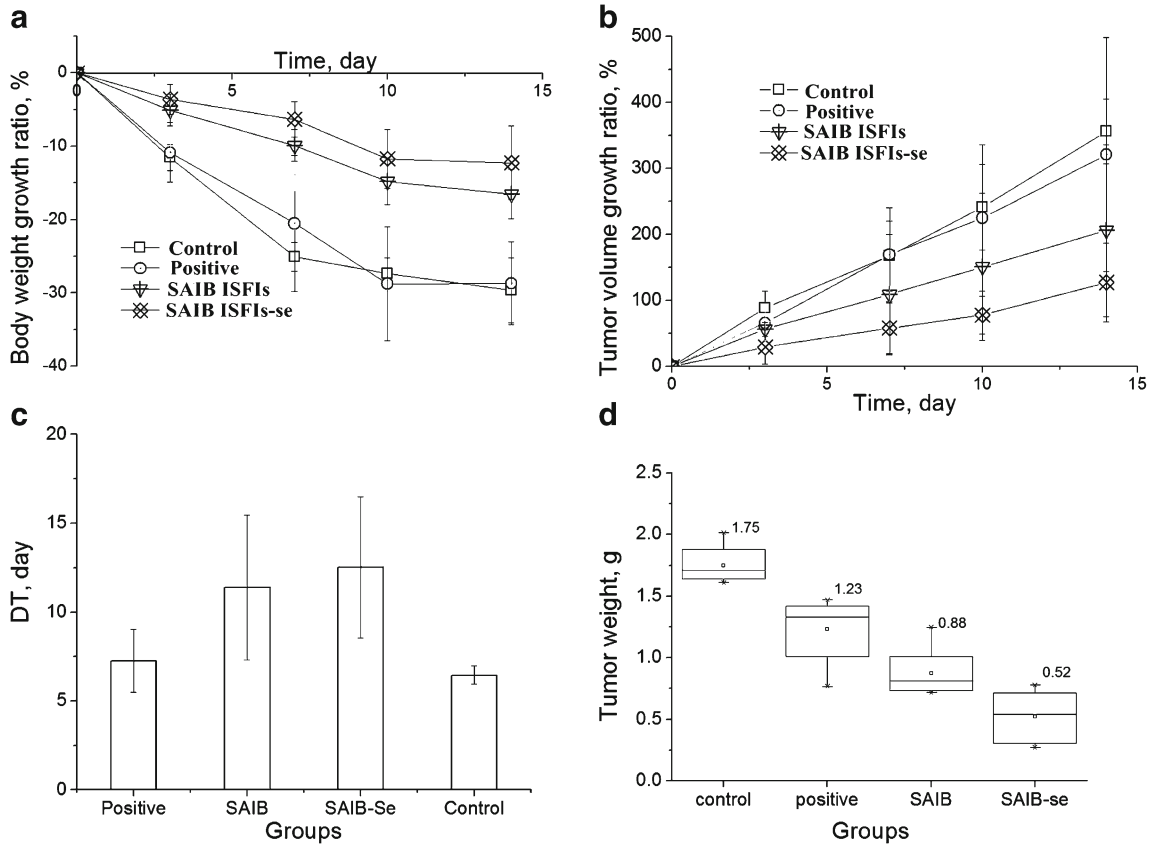

Fig. 10. The anti-melanoma efficiencies of SAIB ISFIs after intratumoral injection to A375.S2 cell bearing mouse. **a** Body weight growth ratio, **b** tumor volume increased ratio, **c** tumor volume doubling time, and **d** final tumor mass

Compared to cuc-loaded PLGA particulate systems as previously reported (1), the ISFIs have superior advantages, such as the ease of manufacturing, good stability, and better anticancer efficiency *in vivo*. In this paper, two parameters, $Q_{3day}/Q_{28\ days}$ (the ratio of the initial drug released to the total drug released) and $AUC_{plasma,0–28\ days}/AUC_{tumor,\ 0–28\ days}$ (the ratio of AUC_{plasma} to AUC_{tumor}), were introduced to elucidate the *in vitro* release and *in vivo* pharmacokinetics profiles of the ISFIs. As shown in Fig. 11, the $Q_{3day}/Q_{28\ days}$ and the $AUC_{plasma,\ 0–28\ days/AUCtumor,\ 0–28\ days}$ of PLGA-based drug delivery system

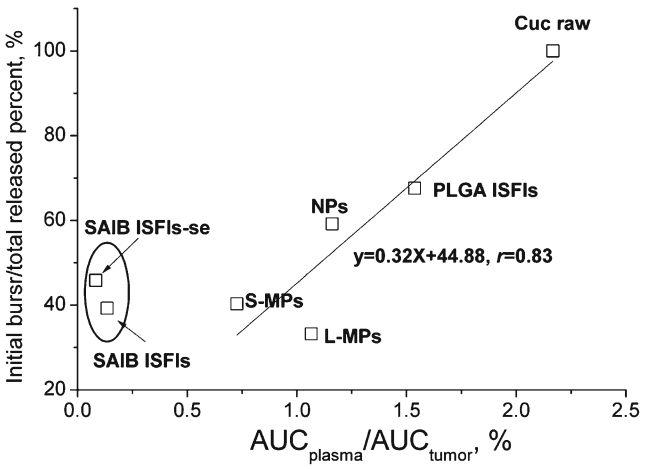

Fig. 11. The relationship between the $Q_{3day}/Q_{28\ days}$ values and AUC_{plasma}/AUC_{tumor} values of various depots

and Cuc-B displayed linear relationship, and higher $Q_{3day}/Q_{28\ days}$ (higher initial burst) corresponded to higher plasma drug concentration (higher systemic toxicities). However, both SAIB ISFI and SAIB ISFIs-se showed high $Q_{3day}/Q_{28\ days}$ and low ratio of $AUC_{plasma,\ 0–28\ days}/AUC_{tumor,\ 0–28\ days}$.

The critical factors that affect the antitumor efficiencies after intratumoral injection are the retention of the preparation inside the tumor, and the drug release profiles from the preparation. The first consideration was the balance of the initial burst release contributed by the depots forms inside or outside the tumor. Higher initial burst will lead to better killing effect to tumor cells, however due to the inevitable leakage of the depots through the pinholes, higher initial burst will also cause higher toxicities (e.g., nanoparticles and PLGA ISFIs). Another consideration was the total drug released during the whole treatment period, for example, large microparticles with very low total drug release could not exert obvious and lasting killing effects (1). In our experiments, small microparticles (S-MPs) and SAIB ISFI presented similar *in vitro* drug release profiles (see Supplementary Figure 2), but SAIB ISFIs displayed better antitumor effect than S-MPs, which may due to its viscous and adhesive properties to reduce the leakage through the pinhole.

CONCLUSIONS

Currently, localized administration of anticancer drugs has become notable and is proved to be an effective strategy for tumor chemotherapy. In this study, two categories of ISFIs based on PLGA or SAIB were investigated for anti-

melanoma efficiencies with intratumoral injection. The results proved that the SAIB ISFIs displayed appropriate pharmaceutical properties, including appropriate release profiles and ease of manufacturing, and expected pharmacokinetic and pharmacodynamic profiles in animal models (higher drug retention in tumor and lower plasma drug concentration, which may result to high therapeutic index). Appropriate drug release profiles (lower initial burst and steady release) and improved retention (minimum leakage from the injection site) could benefit to the antitumor effects of the intratumoral depots. With the development of endoscopic technologies, tumor-localized administration of drug-loaded SAIB ISFIs may provide good potential to treat various types of solid tumors.

ACKNOWLEDGMENTS

We thank Prof. Deng Yihui, Shenyang Pharmaceutical University, for his helpful comments on animal studies, Mr. Tian Han, Zhongshan School of Medicine, SUN YAT-SEN University, for his help on cellular experiments and results explanations, and Prof. Takshi Ikejima, Shenyang Pharmaceutical University, who kindly provided the A375.S2 human melanoma cell.

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